EVALUATION OF SCREENING STRATEGIES FOR THE DETECTION OF MOLECULAR PATHOLOGIES.

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MARIE BOYD

Duncan Guthrie Institute of Medical Genetics

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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

This thesis is dedicated to my mother, In the hope that I have walked the path she would have chosen for me.

And for my Dad, whose well of encouragement, understanding, love and support has been as deep as his wallet !

My love and thanks.

Winning, means winning over the discord in yourself.

Those who have a warped mind, a mind of discord,
have been defeated from the beginning.

Morihei Ueshiba(founder of Aikido)

ABSTRACT

There have been many studies aimed at the determination of the molecular pathologies responsible for genetic disease. Many strategies have been used for detection of base changes in genes which lead to disease and in this study several mutation detection protocols were compared and optimised in order to determine a suitable strategy for the detection of molecular pathologies in large multi-exonic genes. The *HGPRT* gene was used as a model gene and a panel of 10 known mutations present in this gene was used to optimise and compare SSCP, heteroduplex detection on hydrolink gels (HDHG) and chemical cleavage of mismatches (CCM) as a first line screening strategy for pin-pointing the mutation containing exon. Any suspicious exons were then sequenced using automated fluorescent sequencing.

These protocols were then applied to the identification of unknown pathologies in the *HGPRT*, *DMD* and *BRCA1* genes. HDHG was the most successful strategy for detecting changes in larger amplification products where there were fewer exons, while SSCP was most effective when the PCR products were around 200bp in size and where there were a large number of samples to be analysed. Three mutations were identified in the *HGPRT* gene of index cases from Lesch-Nyhan families and the mutation detection strategies coupled with linkage analysis using a tetranucleotide repeat within the *HGPRT* gene, were used to pin-point carriers of the mutations in these families. Four different mutations were found in seven of the breast/ovarian cancer families, including a 2bp deletion which was identified in four unrelated families, indicating the presence of a common Scottish mutation. No mutations were identified in the *DMD/BMD* gene of the individuals who showed no large exon deletion and it may be that the optimum strategy for the detection of mutations in this gene has not yet been identified.

SUMMARY

The overall aim of this study was to optimise and compare several PCR based mutation detection strategies , using the multi-exonic Hypoxanthine guanine phosphoribosyltransferase(*HGPRT*),Duchenne muscular dystrophy (*DMD*) and hereditary breast/ovarian cancer (*BRCA1*) genes as model genes. Scanning strategies were compared using a panel of 10 different *HGPRT* mutations which were present in seven different exons of the gene. These included a 1bp insertion, a 2 bp deletion and eight single nucleotide substitutions. Amplification of the individual exons of the *HGPRT* gene was problematic but after extensive modification of reaction components, in particular the PCR buffer and dNTP concentration and alteration of the annealing temperatures, all exons with the exception of exon 1 were successfully amplified. However the PCR was not particularly robust for a number of exons and the yield and success rate of the amplifications was improved by high level purification of the PCR primers.

Protocols for heteroduplex detection using hydrolink gels (HDHG) and single strand conformation polymorphism analysis (SSCP) were optimised and compared to determine which would be the most effective as a first scanning strategy to indicate the mutation-containing exon. The HDHG protocol had previously only been used for analysis of autosomal genes and primarily for length mutations. The detection efficiency of the hydrolink matrix had also not been evaluated to any great degree. The hydrolink protocol was optimised and developed in this study for an X-linked condition and with the optimised protocol all 10 *HGPRT* mutations were detected with a low false positive rate. In contrast, despite extensive modifications to the gel running conditions, SSCP yielded confusing results and clearly detected only 2 of the original panel of 10 mutations which were present in the smaller sized amplification fragments. This finding was consistent with the reports that SSCP detects mutations at a higher efficiency in small amplification fragments. Further, the HDHG protocol did not require radioactive labelling of the PCR products, negating the need for autoradiography, hence rendering the protocol faster, safer and simpler for analysis of this gene, with the small number of families to be analysed and the small number of exons in the gene.

Heteroduplex detection using hydrolink gels was then utilised as the first scanning stage after multiplex PCR analysis to exclude large length mutations in 13 families with an index case with Lesch-Nyhan syndrome with unknown molecular pathologies. After the mutation containing exon was identified, further information was obtained by an optimised chemical cleavage of mismatch (CCM) protocol and attempted direct sequencing of the amplified mutation containing fragment. Four novel pathological mutations were detected with sequence information being available for three of the mutations. The first mutation involved a base substitution of a guanine for an adenine at base 134 in exon 2 in the index case HGPRT GLA7292 which resulted in an amino acid change of a lysine for an arginine at amino acid 45. This mutation occured in the last bases of exon 2 and is predicted to interfere with RNA splicing. No RNA was available however from this family to confirm this prediction. Another mutation resulted from a 1bp deletion of thymidine at nucleotide 462 in exon 6 of the index case HGPRT GLA7291, which resulted in premature chain termination of the protein 11 amino-acids from the deletion site. In the pedigree HGPRT GLA7294 a large insertion in exon 4 was present which is expected to disrupt the folded conformation of the protein. This exon could not be sequenced due to the exon sequence and no further information could be gathered about this mutation. In a further pedigree HGPRT 10427, a 60 bp insertion was identified in the cDNA from the index case and after sequencing this was identified as the insertion of an ALU element between exons 6 and 7. As the DNA from this index case could not be PCR amplified from this region, the exact site of the insertion was uncertain. The mutation detection strategies coupled with linkage analysis using a tetranucleotide repeat located within the *HGPRT* gene, were subsequently succesfully applied to the designation of carrier status to female relatives of affected index cases in several families.

An identical strategy was used to screen 21 exon/promoter regions of the *DMD* gene from 14 boys who appeared to have no large gene alterations. The PCR had previously been optimised for some of the exons and was easily optimised for the amplification of the remainder. Despite identification of possible base changes using the screening stategies, no conclusive sequence information could be obtained for any of the suspected changes.

Due to the large size of the *BRCA1* gene, the large number of samples to be analysed and the small size of the amplification products designed to amplify the individual exons, it was decided to use SSCP analysis to scan the gene for mutations. Any band shifts identified were sequenced using automated sequencing strategies, and any mutations found were screened for in the remaining samples by allele specific oligonucleotide hybridisation (ASO). 90 family samples were analysed and seven different mutations were found, four of which were pathological in seven families. A 2bp deletion in exon 2 was found in one family(185 del AG>ter 39). This mutation results in a premature termination of the protein at codon 39. This mutation is one of the most common mutations described in the *BRCA1* gene so far in families around the world. A a 1bp insertion in exon 20 (5382 ins C>ter 1892) was found in one family. This mutation results in the production of a truncated protein and has been described in several other unrelated families. A 2 bp deletion in exon 11(2800 del AA>ter 901), was found primarily in one of the *BRCA1* families. This mutation results in premature termination of the protein and was described previously in one American family. After further screening this 2bp deletion was found in four Scottish families who appear unrelated and could indicate the presence of a common Scottish mutation. The remaining fourth probable pathological mutation found was an A>G substitution at base 180. This results in an Ile to Val amino acid substitution in the second amino acid of the RING Finger motif and is suspected to have pathological significance but cannot be confirmed as such in the absence of functional studies on the protein. 50 assumed sporadic ovarian and 100 assumed sporadic breast tumours were also screened for the mutations found and none were identified. Three polymorphisms were also detected which had not previously been described; a G>A substitution in intron 16 which was not thought to interfere with splicing, a T>C substitution at position 4427 in exon 13 which resulted in no amino acid alteration in the protein and a C>T nucleotide substitution at position 2731 in exon 11 resulting in a Pro to Leu amino acid substitution. These changes were presumed to be polymorphisms as the alternate allele was present at high frequencies in both the affected individuals being screened for *BRCA1* mutations and in control DNA and DNA from the DMD and Lesch-Nyhan pedigrees. There was no increase in the frequency of the altered allele in individuals with breast/ovarian cancer compared to other DNA samples indicating that these polymorphisms were probably not significant or exclusive to the *BRCA1*

pathologies.

The mutation detection strategies employed in the analysis of these genes, appeared to be very gene and exon dependent. Different strategies appeared to be better for different genes depending on the exon structure and size and on the number of samples to be analysed. The quality of DNA and hence the success of the PCR amplification in providing good quality amplified DNA as a starting material was also important. No universally appropriate strategy was found for the analysis of all three genes, but the automated sequencing strategy appears the best approach for obtaining good quality sequence.

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LIST OF ABBREVIATIONS

A	adenosine
aa	amino acid
ABI	applied Biosystems inc.
ADRP	autosomal dominant retinitis pigmentosa
ASO	allele specific oligonucleotide
ATP	adenosine tri-phosphate
autorad	autoradiograph
BMD	Becker muscular dystrophy
bp	base pairs
BRCA1	breast cancer gene 1
BSA	bovine serum albumin
C	centigrade
C	cytosine
CCM	chemical cleavage of mismatches
cDNA	complementary copy DNA
CF	cystic fibrosis
Ci	curie
CM	centimetre
CNS	central nervous system
CPM	counts per minute
CRC	cancer research campaign
C-	carboxy terminal
dATP	2,deoxyadenosine 5,-triphosphate
dCTP	2,deoxycytidine 5,-triphosphate
ddh20	double distilled water
ddNTP	2'3' didoexynucleoside
dGTP	2,deoxyguanidine 5,-triphosphate
DGGE	denaturing gradient gel electrophoresis
dITP	2,deoxyinosine 5,-triphosphate
DMD	Duchenne muscular dystrophy
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	2,deoxynucleotide 5, tri-phosphate
dsDNA	double stranded DNA
DTT	dithiothreitol
dTTP	2, deoxythymidine 5,-triphosphate
EDTA	ethylene diamine tetra-acetic acid
et al.	et alia
ETOH	ethanol
g	gram
G	guanodine
GMP	Guanosine monophosphate
h	hour
HDHG	heteroduplex detection using hydrolink gels
HEX	hexaminidase
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
HPLC	high performance liquid chromatography
IQ	intelligence quota
IMP	inosine monophosphate
K	1000 rev/min

kb	kilo-base
kd	kilo-daltons
l	litre
LOH	loss of heterozygosity
M	molar
mA	mili amp
Mb	mega-base-pair
MDE	mutation detection enhanced
MgCL2	magnesium chloride
min	minute
ml	mili-litre
mm	mili-meter
mRNA	messenger RNA
n	nano
NaI	sodium iodide
ng	nano gram
nm	nano meter
NaCl	sodium chloride
NaOH	sodium hydroxide
OD	optical density
oligo	oligonucleotide
OPC	oligonucleotide purification cartridge
OsO_4	osmium tetroxide
p	pico
pm	pico moles
P	phosphorous
PCR	polymerase chain reaction
PRPP	phosphoribosyl-1-pyrophosphate
Rb	retinoblastoma
RNA	ribonucleic acid
RNASE	ribonuclease
RFLP	restriction fragment length polymorphism
RT	room temperatue
S	sulphur
SCK	serum creatine kinase
SDS	sodium dodecyl sulphate
secs	seconds
ssDNA	single strand DNA
SS	single strand
SSCP	single strand conformation polymorphism
STR	short tandem repeat
T	thymidine
Taq	Thermus aquaticus
TBE	tris. borate.EDTA buffer
T.E.	tris/ EDTA buffer
TEMED	tetramethlyammonium aceticacid
ter	terminate
TMAC	trimethylammonium chloride
TONI	trapped oligonucleotide incorporation assay
Tris	tris(hydroxymethyl) aminoethane
tRNA	transfer RNA
UV	ultra violet
WT	Wilm's tumour

w/v	weight per volume
Xp	short arm of X chromosome
Xq	long arm of the X chromosome

CHAPTER 1

INTRODUCTION

1.1 Genetic Disease.

Genetic disease has become increasingly important in recent years as more infections are becoming controllable . Therefore, research is being aimed towards the elucidation of molecular pathways and how their breakdown can contribute and cause disease. From the knowledge of the central dogma that DNA encodes for RNA which encodes for a particular protein it is obvious that any change to the DNA sequence will have an effect on the encoded protein. It is on this basis that genetic disease must be examined, to understand and to be able to deal with genetic disease on a clinical basis.

Many genetic diseases are a result of small alterations involving single genes or parts of genes and the effects of these changes are often difficult to spot and to understand. There are many human genes which have been mapped and their role in the biological pathways and their breakdown understood, however there are still many diseases thought to have a genetic component where no specific gene has been attributed . With such a wide variety and high prevalence of genetic disease, and with the lack of medical tools available in their treatment , it has become necessary to be able to assign carrier status, to prenatally diagnose affected pregnancies and to identify carriers of cancer predisposing genes to aid disease management.

Many diseases involving enzyme defects can be diagnosed biochemically although the enzyme levels for affected individuals, carriers and the normal population often overlap rendering the results difficult to interpret. Other traditional methods of prenatal diagnosis and carrier detection involve linkage analysis using polymorphic markers, but these methods are not always suitable or the results informative. It is partly because of this and because information is being sought about the exact molecular nature of these conditions at the protein and DNA levels that there is an increasing demand for information about the exact nature of the DNA and protein alterations in genetic diseases.

There are many strategies for investigation of the molecular pathologies of genetic diseases and this study is aimed to compare several different mutation detection techniques in three multiexonic hereditary diseases, Lesch-Nyhan syndrome, Duchenne /Becker muscular dystrophy and inherited early onset breast / ovarian cancer.

1.2 Lesch-Nyhan Syndrome.

Lesch-Nyhan syndrome is an X-Linked recessive disease, with a birth incidence of 1 in 100,000 male births, (Kelley et al., 1983) which is caused by deficiency of an enzyme hypoxanthine-guanine phospho-ribosyltransferase (HGPRT) . The genetic features of the disease make it highly amenable to study because of its position on the X chromosome as is shown in figure 1.1, rendering the mutant alleles hemizygotic in male cells and functionally hemizygotic in female cells as a consequence of X-inactivation. The hereditary pattern of the condition is therefore simpler to follow in pedigree analysis as one in two male progeny are affected and one in two female progeny are on average, carriers. The gene known to be defective in this condition, the *HGPRT* gene, is well characterised as since its initial description in the late 1960's (Lesch and Nyhan, 1964), HGPRT deficiency has been the subject of intensive investigation.

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1.2.1 The Clinical Features of Lesch-Nyhan Syndrome.

There are two distinct clinical syndromes associated with deficiency of the human HGPRT enzyme. Complete deficiency of enzyme activity is associated with an X-linked recessive condition , Lesch-Nyhan syndrome (Lesch and Nyhan, 1964), while partial deficiency of enzyme activity results in hyperuricemia and a form of gouty arthritis (Kelley and Wyngaarden, 1983). However a complete spectrum of HGPRT activity levels from zero to 30% have been reported.

Lesch-Nyhan syndrome was first described as a familial disorder of uric acid metabolism and central nervous system dysfunction and the specific enzyme defect was subsequently reported by Seegmiller in 1967. The symptoms include spastic paralysis, mental retardation and choreoathetosis which becomes increasingly marked with age (Lieber et al., 1973) and bizarre , aggressive behaviour , particularly in the form of

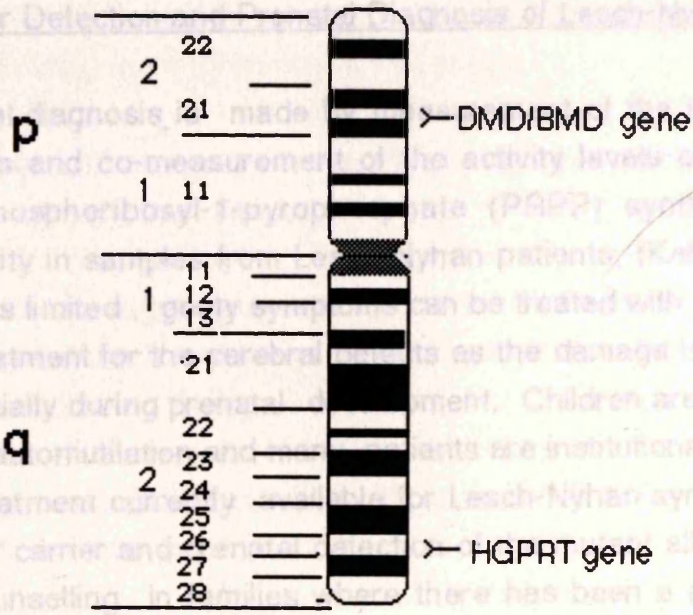


Figure 1.1. Location of the HGPRT and DMD genes on the X-chromosome.

biting,scratching and self-mutilation (Letts et al., 1975, Francke et al., 1976). In affected boys developmental delay and all the symptoms of gouty arthritis may develop. The symptoms generally manifest themselves in the first year of life and the aggressive tendencies become apparent usually in the third year of life with patients not usually surviving beyond five years.

1.2.2 Carrier Detection and Prenatal Diagnosis of Lesch-Nyhan Syndrome.

Initial diagnosis is made by measurement of the HGPRT levels in erythrocytes and co-measurement of the activity levels of other enzymes such as phosphoribosyl-1-pyrophosphate (PRPP) synthase which has raised activity in samples from Lesch-Nyhan patients, (Kelley et al., 1978). Treatment is limited , gouty symptoms can be treated with drugs , but there is little treatment for the cerebral defects as the damage is thought to have occured initially during prenatal development. Children are often restrained to prevent automutilation and many patients are institutionalised. Due to the lack of treatment currently available for Lesch-Nyhan syndrome there is a demand for carrier and prenatal detection of the mutant allele, coupled with genetic counselling in families where there has been a previous affected child.

Due to Lyonisation (Lyon, 1961), biochemical analysis of carrier status depends on the ability to distinguish between the two populations of cells from a cell culture, from a skin biopsy or by measurement of HGPRT activity in a single hair root (Gartler et al., 1971;Halley and Heukels-Dully, 1977; Watts et al., 1982). These methods have given some results, however the measurements must be interpreted on the basis of the individual families and often interpretation is very difficult and time consuming and direct detection of the responsible mutations at the DNA level would be preferable.

1.2.3 The HGPRT Gene Structure and Function.

The *HGPRT* gene encodes an enzyme which belongs to the family of phosphoribosyltransferases. These enzymes are found in all eukaryotic organisms. HGPRT catalyses the condensation of 5'phosphoribosyl-1-pyrophosphate (PRPP), and the purine bases hypoxanthine and guanine in the formation of 5'IMP and 5'GMP respectively (Buchanan and Hartman, 1959, Buchanan, 1960, Kelley et al., 1983). This pathway is shown in figure

1.2. These vital enzymes catalyse the salvage of hypoxanthine and guanine which are precursors of DNA and RNA and often act as coenzymes in enzymatic reactions, thus providing cells with an alternative to the energy expensive process of de-novo synthesis of nucleotides. HGPRT plays a critical role in the maintenance of intracellular purine nucleotide pools in cells that have a decreased ability to synthesise new nucleotides (Fontenelle and Henderson, 1969). 90% of free purines in humans are recycled indicating the importance of this enzyme.

The HGPRT enzyme is found in all human tissues at low levels as a cytoplasmic protein that accounts for approximately 0.005-0.04% of total cellular protein. HGPRT activity is greatest in the basal ganglia of the brain and in the testes suggesting that de novo synthesis of purines in these areas is very low and that these regions are probably very dependent on the salvage pathway (Kelley et al., 1969, Howard et al., 1970, Allsop and Watts, 1980,). These tissue differences suggest a different role for the enzyme in different tissues and the dependence of these certain tissues on HGPRT activity is concordant with the disease pattern and pathology.

Wilson et al., (1982), determined the amino acid sequence of the enzyme in human erythrocytes by cleaving the protein into overlapping peptide fragments and subjecting these to Edman degradation . The functional enzyme is 217 amino acids long after the post translational cleavage of the amino terminal methionine and has a molecular weight of 24,470 (Wilson et al.,1982). The native HGPRT is a multimeric enzyme composed of identical subunits. Holden and Kelley, (1978), investigated the native structure of the enzyme using cross-linking agents and these studies coupled with isoelectric focusing (Arnold and Kelly, 1971) predicted that the native HGPRT enzyme may exist as a tetramer. HGPRT is one of many related phosphoribosyltransferases which are found in many organisms and many are functionally and structurally similar with sequence comparisons between mouse, hamster and human cDNA indicating a high degree of sequence homology (Chinault and Caskey, 1984). On comparison of amino acid sequences correlation was noted in a 120 aa residue region, suggesting the presence of a common structural domain and secondary structure predictions of this region indicate the presence of a dinucleotide with a characteristic $\beta-\alpha-\beta$ pattern (Argos et al., 1983). This type of

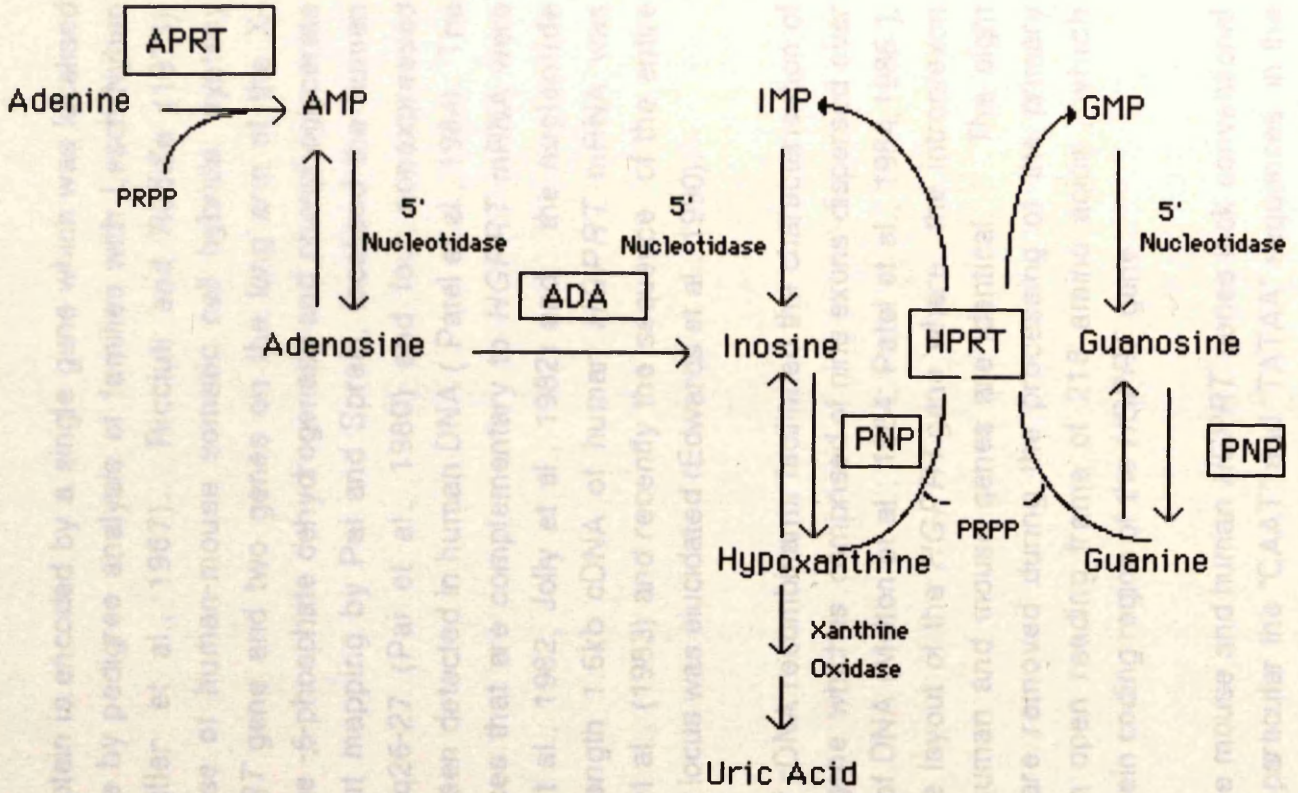


Figure 1.2. The purine synthetic pathway. APRT - adenine phosphoribosyltransferase; PRPP - 5'phosphoribosyl-1-pyrophosphate ADA - adenosine deaminase; PNP - purine nucleoside phosphorylase HPRT - hypoxanthine guanine phosphoribosyltransferase.

structure has been identified in many different enzymes and in the HGPRT enzyme is thought to be the nucleotide binding domain.

The human protein is encoded by a single gene which was localised to the X-chromosome by pedigree analysis of families with Lesch-Nyhan syndrome (Seegmiller et al., 1967). Ricciuti and Ruddle (1973), demonstrated by use of human-mouse somatic cell hybrids, synteny between the *HGPRT* gene and two genes on the long arm of the X-chromosome , glucose -6-phosphate dehydrogenase and phosphoglycerate kinase . Subsequent mapping by Pai and Spraki, localised the human HGPRT gene to Xq26-27 (Pai et al., 1980) and four nonexpressed pseudogenes have been detected in human DNA (Patel et al., 1984). The cloned DNA sequences that are complementary to *HGPRT* mRNA were isolated (Brennand et al., 1982; Jolly et al., 1982) and the nucleotide sequence of a full length 1.6kb cDNA of human *HGPRT* mRNA was determined by Jolly et al., (1983) and recently the sequence of the entire human *HGPRT* gene locus was elucidated (Edwards et al., 1990).

The isolation of cDNA recombinants facilitated the characterisation of the human *HGPRT* gene which is comprised of nine exons dispersed over approximately 44 kb of DNA (Melton et al., 1984; Patel et al., 1984,1986). Figure 1.3 shows the layout of the *HGPRT* gene where the intron/exon boundaries for the human and mouse genes are identical. The eight intronic sequences are removed during the processing of the primary transcript to yield an open reading frame of 218 amino acids , which correspond to the protein coding region of the *HGPRT* gene .

Interestingly, the mouse and human *HGPRT* genes lack conventional promoter elements in particular the "CAAT" and "TATAA" sequences in the 5' flanking sequences (Melton et al., 1984; Patel et al., 1986) . Both human and mouse 5' non-coding regions are particularly GC rich and approximately 80% of the 250 base pair sequence 5-prime to the initiation codon are G or C bases. The control mechanisms are thought to involve the binding of promoter specific transcription factors which bind to copies of a hexanucleotide CCGCCC sequence which is present and methylation of cytosine residues is also thought to be involved in gene expression through its role in X- inactivation (Wolf et al., 1984). Deletion analysis suggests that additional factors in the promoter may play a role in transcriptional control.

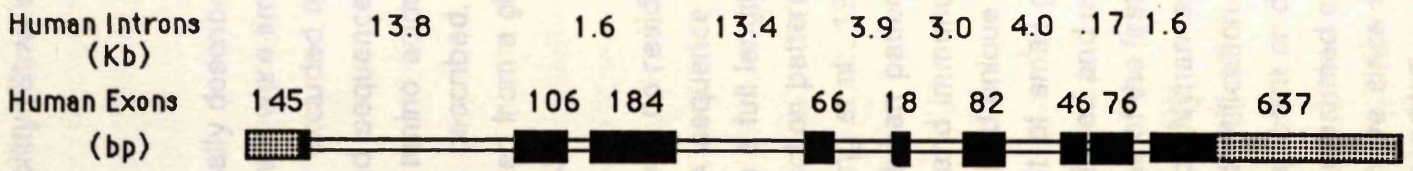


Figure 1.3. Structural organisation of the HGPRT gene. The exons are represented by the boxed areas.

The molecular basis of the tissue variability in expression of the *HGPRT* gene has been addressed but the exact signals responsible for the elevated levels in the brain have not been identified. Studies using transgenic mice that express the human *HGPRT* minigene suggested that it is sequences within the human *HGPRT* transcript (cDNA) which influences its expression, probably via increased mRNA synthesis or stability (Stout et al., 1985).

1.2.4 Mutations in the *HGPRT* Gene.

Mutations in the *HGPRT* gene were initially described on the basis of altered protein or enzymatic parameters and thus were limited to mutations which resulted in residual HGPRT activity and excluded most patients with Lesch-Nyhan syndrome. Once the amino acid sequence was elucidated mutations could be found in terms of altered amino acids in patients with residual protein and one of the first mutations described, $HGPRT_{Toronto}$, was isolated from a patient with gout and resulted from a glycine for arginine substitution at amino acid 50 (Wilson et al., 1983).

The analysis of Lesch-Nyhan patients with no residual enzyme, was first carried out by directly looking at the DNA sequence using restriction enzyme digestion and Southern Blotting using a full length *HGPRT* probe (Yang et al., 1984) and looking for altered restriction patterns. Many *HGPRT* mutations were identified by this approach (Yang et al., 1984; Wilson et al., 1986). Genetic heterogeneity among Lesch-Nyhan patients was apparent and studies using Southern, Northern, kinetic and immunoquantitative data showed that most patients with the condition had unique genetic features with many of the phenotypes being the result of small genetic changes (Wilson et al., 1986). Methods were then developed and used to attempt to identify the exact nature of these alterations. One of the first methods used in the search for molecular pathologies in Lesch-Nyhan syndrome was an RNase cleavage method which resulted in identification of five sequence changes including two deletions and three small point or deletion mutations (Gibbs and Caskey, 1987). This technique was presumed only to detect 50% of possible mutations and further techniques have since been used based on PCR based methods to detect many further *HGPRT* mutations (Davidson et al., 1988,1989, 1991; Edwards et al., 1989; Gibbs et al., 1989; Gibbs et al., 1990; Marcus et al., 1992; Yamada et al., 1992; Steingrimsdottir et al., 1992; Sege-Peterson et al., 1992; Tarle et al., 1992).

1.3.1Duchenne/Becker Muscular Dystrophy.

The muscular dystophies represent some of the most serious human genetic diseases . X-linked muscular dystrophies are of the severe or mild types known as Duchenne and Becker muscular dystrophy (DMD and BMD) respectively. Duchenne muscular dystrophy (DMD), was first studied by Duchenne in 1868 (Duchenne, 1868) and the clinical features were later described by Gowers in 1879. DMD is an X-linked recessive trait with a birth incidence of one in 3000-3500 males (Emery, 1988).

The clinical features of DMD and their relative importance is largely age-related. In over half the affected boys, walking and general development of motor functions is delayed and in some boys there is a more general delay in development. An abnormal gait and general muscle weakness can be noted and calf and other muscle enlargement occurs due to an excess of adipose and connective tissue. Later, other clinical signs become prevalent including slight facial weakness and the intercostal muscles become affected. These symptoms lead to very apparent physical features, but In almost all DMD patients it is cardiorespiratory problems related to diminished respiratory capacity which are the principal causes of death. The milder Becker muscular dystrophy (BMD), has a later onset and a milder course and was first recognised by Becker in 1955. Age at death is also delayed in BMD and the other clinical symptoms described occur less frequently.

1.3.2 Diagnosis of DMD/BMD.

Diagnosis of DMD can usually be made by assessment of the clinical features and is aided when a family history of the disease exists. Diagnosis can be supported by examining the serum levels of the muscle enzyme serum creatine kinase (SCK) and by muscle biopsy. Ebashi et al., (1959) and Dreyfus et al., (1960), first noted that the activity of this enzyme is elevated in DMD patients. The enzyme escapes from the damaged muscle into the serum and this accounts for the increased levels.

Significant changes in muscle pathology are present from birth in an affected individual with DMD before the occurrence of obvious clinical symptoms (Cullen and Mastaglia, 1980). The appearance of muscle

degeneration in BMD is very similar and these changes can be identified and used as suitable diagnostic indicators in both conditions.

As with other X-linked disorders, female carriers of the abnormal gene are generally healthy and the occurrence of DMD/BMD in a female is very rare. In approximately 2.5% of carriers a small degree of muscle weakness is apparent due to a variation in X chromosome inactivation. The severity of the disease in these females varies and these manifesting carriers usually have onset later in adult life with asymmetric wasting and weakness, an elevated SCK and muscle biopsy suggestive of DMD. Progression of the disease is generally slower than in males.

1.3.3 Genetics of DMD/BMD.

DMD shows a X-linked recessive pattern of inheritance with hemizygous males showing full penetrance. Most studies give an incidence of 1:3000-3500 male births . The actual new mutation rate for DMD is estimated to be 10^{-4} mutations per gene per generation and 1/3 of all patients should result from a new mutation, if the male and female mutation rates for recessive X-linked lethal disorders is equal and the reproductive viability of the patient is zero (Haldane, 1935). This high new mutation rate is also thought to reflect the large size of the *DMD* gene.

1.3.4 The *DMD/BMD* Gene and Gene Product.

Mapping and molecular genetic studies indicated that DMD/BMD is a result of mutations in a huge gene that encodes the protein dystrophin (Hoffman et al., 1987). The gene was mapped to the Xp21 region of the X-chromosome (figure 1.1), by a combination of techniques. (Boyd et al., 1986; Davies et al., 81, 83; Kingston et al., 1983, 1984; Wilcox et al., 1985; Francke et al., 1985). These studies coupled with the detailed positional cloning and molecular analysis (Kunkel et al., 1985; Ray et al., 1985) localised the *DMD* gene to the short arm of the X chromosome and the chromosomal region involved many kilobases of DNA. The cDNA for the *DMD* gene was isolated by Koenig et al. (1987) by looking at cross species conservation and was shown to represent 14kb of DNA containing more than 65 exons with the most recent data suggesting more than 78 exons. The gene encoded a protein product named dystrophin.

Dystrophin (Hoffman et al., 1987), is a 3685 amino acid hydrophilic protein with a predicted molecular weight of 427kd. It has four distinct domains which share many features with the proteins spectrin and α-actinin (Hammonds et al., 1987). The function of the dystophin protein was originally speculated to be due to its linkage to an integral membrane glycoprotein in the sarcolema which acts as a dystrophin receptor (Campbell and Kahl, 1989). Dystrophin is now thought to be associated with many glycoproteins in the membrane and appears to serve as a specialised link between the actin cytoskeleton and components external to the sarcolemal membrane (Ervasti and Campbell, 1991).

Dystrophin transcription is apparent at different levels in many different tissues, being most prevalent in skeletal muscle and lower in smooth muscle and in many other tissues including the brain. (Chelly et al., 1988). Expression of the large dystrophin gene is under elaborate transcriptional and splicing control. At least 5 independent promoters specify the transcription of their respective alternative first exons in a cell specific and developmentally controlled manner and alternative transcripts have been identified in many different tissues. There appear to be many dystrophins and it is likely that dystrophin may have many different functions in different tissues.

1.3.5 Detection of the Mutant Gene.

As with other X-linked disorders, detection of the mutant gene is problematic due to the absence of symptoms in most carriers. When a pedigree is informative,when a woman has two affected sons or one affected son and an affected close male relative, she can be designated an obligate carrier, otherwise the risk can be clarified by estimating the levels of SCK which are known to be elevated in some mothers of boys affected with DMD, (Emery, 1965). However, other factors can alter SCK values in females including exercise (Hudgson et al., 1967), age (Lane and Roses, 1981) and pregnancy, making carrier detection based on SCK values alone unreliable. The best method for carrier assignment is to assess the probability carrier status using Bayesian statistics where a combination of pedigree data, SCK data and DNA analysis would be considered.

Spelling

In the past the only method of ensuring that no male children with DMD/BMD were born to females at risk was to carry out selective termination of all male fetuses after fetal sexing. Now fetal DNA can be analysed by linkage analysis using closely linked markers or gene specific probes. However the large size of the gene makes recombination events between the gene and the markers possible so it is more effective to use a number of markers and to use intragenic markers. Linkage analysis has further problems in that it is necessary to have DNA from key family members who may not always be available , that non-paternity may exist in one or more family members and that in approximately 1/3 of all cases of DMD are estimated to be due to a new mutation (Davie and Emery, 1978). Further complications arise due the similar clinical patterns of other conditions like DMD which have different patterns of inheritance.

It is therefore obvious that detection of the molecular pathologies responsible for the disease in individual families, would be the best method for definitive prenatal diagnosis and assignment of carrier status. Sporadic cases could then be identified and mothers and sisters can be excluded as carriers. The advent of PCR technology also negates the need for large quantities of good quality DNA to be available and where the index cases are dead small quantities of DNA from sources such as paraffin embedded muscle samples may be used.

1.3.6 The Molecular Pathologies Involved in DMD/BMD.

The very large size and complexity of the dystrophin gene makes the detection of molecular pathologies in the gene a difficult and time consuming task. The entire coding region spans 2.4 million bases which includes many large introns. Deletions and duplications account for approximately 70% of mutations found (Koenig et al., 1987; Hodgson et al., 1989; Hu et al., 1989) and smaller mutations such as point mutations account for the remaining 30% of cases and appear to cause alteration of consensus splice site sequences and in premature translational termination or mutations in promoter elements and hence absence of a functional protein. BMD which is less severe is usually due to rearrangements where the translational reading frame is maintained (Monaco et al., 1988; Koenig at al., 1989).

The region of the protein affected determines the clinical severity of the disease (Koenig et al., 1989). A deletion of a large part of the amino-terminus may lead only to the BMD phenotype while deletions in the carboxy-terminal domain tend to lead to DMD (Bulman et al., 1991). The proximal rod region appears to be relatively dispensable as deletions here leads to milder phenotypes and typical BMD (Ahn and Kunkel, 1993). Large deletions and insertions can be picked up using cDNA probes by Southern blotting (Gilgenkrantz et al., 1989), but these probes do not detect all of the deletions. In 1988, Chamberlain et al., developed a good detection system based on PCR where sequences flanking the exons were utilised to screen for the presence or absence of the exon (Chamberlain et al., 1988).

The search for smaller pathologies has proved to be more difficult. Several attempts to look for these in the 30% of cases with no apparent large alterations have been made. These have involved the utilisation of several techniques including looking at shifts in the electrophoretic mobility of exons in acrylamide gels, by CCM analysis of individual exons, direct sequencing and by looking for the formation of heteroduplexes in heterozygous individuals (Bulman et al., 1991; Clemens et al., 1992; Kilimann et al.,1992; Roberts et al., 1992; Saad et al., 1992). More recently a protocol involving the *in vitro* transcription and translation of portions of the DNA, amplified by PCR using a primer with T7 polymerase initiation sights, has been used to look for premature stops in the gene, induced by frameshift mutations(Roberts et al., 1991;Roest et al., 1993; Van Der Luijy et al., 1994)).

1.4.1 Inherited Breast and Ovarian cancer.

Many cancers can be caused by mutations in cancer predisposing genes. Breast cancer is a common malignancy, affecting approximately one in 8-12 women during their lifetime. Many factors have been identified which increase a womans' lifetime risk of developing the disease and in particular a positive family history has been identified as a major risk factor in early onset breast/ovarian cancer. Segregation analysis of families with multiple affected individuals and linkage analysis confirmed that one or more autosomal dominant genes appeared to account for 5-10% of all breast cancers (Newman et al., 1988, Clause et al., 1991). One of the genes responsible(*BRCA1*) was mapped to chromosome 17q21 in 1990 (Hall et al., 1990), and numerous other groups confirmed this linkage in families with

breast cancer and in particular with early onset breast and ovarian cancers. However, the great majority of breast and ovarian cancers occuring in the population are due to acquired mutations , but despite the small percentage of affected patients being due to inherited mutations of the *BRCA1* gene, it is a common genetic disease that allows the possibility for genetic counselling and pre-disease diagnosis.

1.4.2 The *BRCA1* Gene.

The gene responsible for approximately 45% of inherited breast cancer and 80% of inherited breast and ovarian cancer, the *BRCA1* gene, was identified in 1994 by Easton et al,. (Easton et al ., 1994). Risks of developing breast and or ovarian cancer after inheriting an aberrant *BRCA1* gene, are extremely high, exceeding 50% before age 50 years and reaching 80% by age 65 years(Easton et al., 1993) . More recently a second succeptability gene, *BRCA2* was localised to chromosome 13q12-13 (Wooster et al., 1994). Alterations in other genes have also been shown to be responsible for inherited breast cancer, including many of the common oncogenes such as *ras*, the *p53* gene (Malkin et al., 1990), and the androgen receptor gene (Wooster et al., 1992), mutations in which can lead to breast cancer in males.

The *BRCA1* gene encompassed 24 exons, 22 of which are thought to be coding, which results in a cDNA encoding a 1863 amino-acid protein . The function of this protein is not yet understood as much of the gene shows no homology to any other known protein. The exception to this is a 126 nucleotide sequence at the amino terminus, which encodes a RING finger motif (Lovering et al., 1993) , a motif found in other proteins that interact with nucleic acid and/or form protein complexes. This may suggest a role for the BRCA1 protein in DNA transcription although no studies to date have yet confirmed or disproved this theory.

1.4.3 Mutations in the *BRCA1* Gene.

The frequency of *BRCA1* germline mutations in the population has not yet been determined but the initial screen for germ-line mutations has revealed a mixture of frameshift, nonsense and regulatory mutations resulting from small and large deletions, insertions and substitutions (Miki et al 1994., Futreal et al., 1994,. Friedman et al., 1994 ; Castilla et al 1994, Shattuck-Eidens et al.,1995; Hogervorst et al.,1995). The initial statistics suggest a population carrier frequency of 1/2000 to 1/5000 but these reports are probably an over-estimate. These mutations are thought to produce both truncated and abnormal proteins as well as producing alterations in the regulatory mechanisms leading to abnormal and absent transcripts from the mutant allele. The mutations appear along the length of the gene in contrast to other cancer predisposing genes such as *p53* and hence no apparent clustering of mutations has been reported. However a good proportion of the pathologies appear in exon 11 which encodes for the majority of the protein and to date three mutations appear to be relatively common in unrelated families, a 1bp C insertion in exon 20, a 2bp AG deletion in exon two and a splice mutation in intron 6. Different populations appear to show other more common mutations and an AA deletion in exon 11,highlighted in this study which appears to be relatively common in the Scottish population. Before the cloning of the *BRCA1* gene, there was an expectation that somatic mutations in the gene would be found to contribute to tumourgenesis in the far more common sporadic forms of the disease. This was predicted due to the observation that loss of heterozygosity (LOH), the molecular genetic hallmark of tumour suppressor gene inactivation, was apparent in sporadic breast and ovarian cancers in the region of the *BRCA1* gene. A large percentage of sporadic tumours contain allelic deletions at the 17q locus and hence it was a surprise when groups such as Futreal et al (1994), could find no mutations in these sporadic tumours in the *BRCA1* gene. This would not be surprising in the context of other genes such as the *WT1* tumour suppressor gene where the gene is mutated in most familial cancers but rarely in sporadic disease. Further, additional mapping studies indicated the possible existence of other tumour suppressor genes in the *BRCA1* region (Jacobs et al., 1993, Saito et al., 1993). However two groups have reported the presence of mutations in the *BRCA1* gene in at least 5 sporadic ovarian tumours (Merajver et al., 1995, Hosking et al., 1995) and further reports are being made of mutations found by other groups in

sporadic ovarian tumours. Again there appears to be no clustering of these mutations as they appear along the length of the gene. There has been no reports however of mutations being identified in the *BRCA1* gene of sporadic breast cancers which may indicate that this gene plays no role in the sporadic form of this disease.

1.5 Mutation Detection Methods.

The ability to detect changes in human DNA is fundamentally important in the examination and understanding of the molecular pathologies which underly genetic disease. This can be carried out by the use of indirect methods for following the segregation of linked markers with the disease loci or by directly looking for base changes in protein coding genes.

1.5.1 Indirect Mutation Detection.

The detection of base changes was first done by use of restriction endonucleases coupled with the Southern blotting technique to detect the loss or gain of restriction enzyme recognition sites (Southern, 1975). This approach has several limitations, primarily that not all single or multiple base mutations altered a restriction enzyme recognition site and many enzymes were not commercially available or would have produced unacceptably small or large fragments . The resolution of this technique is also not suitable to pinpoint small differences in fragment length, but despite these problems many genetic diseases can be diagnosed by this method by virtue of their linkage to known restriction site polymorphisms. These polymorphic sites are inherited in a codominant fashion and are either intragenic or extragenic and co-segregate with the gene loci. By following the inheritance pattern of the fragments generated by restriction enzyme digestion of the DNA from a family, these differences in DNA sequence can be used as markers. In many cases however, the segregation of the linked marker in certain families is uninformative because DNA from key relatives is unavailable or because of marker homozygosity. Misdiagnosis can also occur because of recombination between the marker and the mutation. Other problems arise from non-determination of phase and from non-paternity. Thus many factors determine the success or failure of this technique including how many family members are available, the quantity

and quality of DNA available and on how close the marker is to the gene of interest.

Base changes which do not alter a restriction enzyme site can be used as markers by detection with differential hybridisation of specifically designed synthetic oligonucleotide probes, which can be made to have a sequence corresponding to a known polymorphic allele sequences. Stringent hybridisation is carried out on family members to screen for the presence of the different alleles. However this method depends on having available the exact sequence of the region and could be expensive if a large number of alleles exist and a large number of marker sites are being examined.

As well as using conventional RFLP'S for linkage analysis, tandemly repeated DNA sequences within the human genome can be used. The human genome contains several classes of tandemly repeated DNA which are frequently polymorphic (Savatier et al., 1985). One particular class of these are the short tandem repeats (STR) polymorphisms with mono-, di, tri, and tetranucleotide repeat motifs. (Litt and Luty, 1989; Weber and May, 1989; Boylan et al., 1990; Econamou et al. , 1990; Zulani and Hobbs, 1990; Edwards et al., 1991). These STR's exhibit Mendelian codominant inheritance and are highly polymorphic due to extensive variation in the number of repeat units, and many of these STR's have heterozygosity values of up to 90%.

The STR's can be easily amplified with PCR and allele lengths can be precisely resolved to single bases by analysis of 100-500bp amplified fragments on polyacrylamide sequencing gels. The frequency of particular STR's is sufficiently high that it should be possible to identify a STR locus in most genomic segments (Edwards et al., 1991). The estimated frequency of the 44 possible unique trimeric and tetrameric STR's in the genome is estimated to be 1 STR /10kb. Edwards et al., (1991), describe a method by which DNA segments flanked by an STR locus may be amplified and sequenced directly thus allowing oligonucleotide primers to be designed for their amplification.

The longer and more complex trimeric and tetrameric STR motifs seem to provide a greater amplification fidelity than for the smaller (AC)

repeats. STR loci found in the primate sequences of GeneBank indicate that trimeric and tetrameric STR's are found within coding, genic (introns and flanking sequences) and extragenic regions of the genome. Only trimeric STR's were found within coding sequences.

These STR repeats have several advantages over traditional RFLP linkage analysis utilising blotting techniques. Of particular use in carrier detection in the *HGPRT* gene is a polymorphic (AGAT) repeat with 9-16 copies, located within the human *HGPRT* gene (Edwards et al., 1991). This repeat would be a potentially ideal intragenic, polymorphic, reliable marker with which to quickly and easily trace the path of an affected X chromosome in a family with Lesch-Nyhan syndrome. Further, this linkage is not likely to be hindered by recombination between the marker and the gene due to its position within the *HGPRT* gene and only a small quantity of DNA is needed for analysis by PCR amplification . This has the potential to be used in families to quickly exclude females as carriers and will be of particular value where no molecular pathology has been detected.

Cases of non-paternity may interfere with this analysis and it will not identify new mutations in index cases allowing a number of false positive carrier risks to be assigned. Therefore although this protocol may have its advantages in this study, ideally direct mutation detection strategies would pinpoint the exact presence or absence of molecular pathologies.

1.5.2 Direct Mutation Detection.

There are many methods available for the direct detection of mutations in human genes. Some rely on the differential electrophoretic mobility of DNA sequences differing by as little as a single base pair or in the differential melting temperatures of DNA heteroduplexes and homoduplexes. Further methods rely on the reaction of non-paired nucleotides in heteroduplexes between affected and non-affected patients DNA or RNA with certain enzymes or chemicals, or on direct comparison of length ,sequence conformation of DNA or in differential hybridisation of oligonucleotides to normal and mutant sequences. Some of the most useful of these methods which will be used in this study and will now be examined.

1.5.3 The Polymerase Chain Reaction.

In vitro DNA amplification using the polymerase chain reaction (PCR) (Saiki et al., 1985,88) has revolutionised molecular genetics. The procedure was first documented by Saiki et al., in 1985 and proposed a method which would circumvent the need for time consuming cloning strategies. Since then a wide variety of different methods which can have far reaching applications in DNA research have been reported. PCR allows the specific *in vitro* production of multiple copies of defined fragments of DNA. The reaction is highly specific and sensitive and only one copy of a sequence in a single cell is theoretically required for amplification to occur. The original theory behind the procedure was described by Kleppe et al., (1971).

Amplification involves a primer mediated enzymatic amplification of target sequences in genomic DNA by repeated cycles of (1) heat denaturation of the double stranded template, (2) primer annealing and (3) extension of the annealed primer with DNA polymerase. In each cycle the number of copies of the target sequence is exponentially doubled, resulting in exponential amplification.

The specificity of the amplification is determined by the sequence of the short oligonucleotides which hybridise to different strands of a DNA template in a relative orientation. The template can be a small amount of sequence such as a discrete molecule or a small sequence embedded in a vast sequence such as the human genome, a single-stranded DNA molecule or an RNA molecule. The product of the reaction is a discrete double-stranded molecule with termini corresponding to the 5' end of the primers used. The technique became more widely used after the introduction of thermostable Taq DNA polymerase, which relieved the necessity of adding new enzyme after each denaturation step so allowing automation of the procedure.

There have been many protocols suggested for the improvement of the PCR protocol such as the use of cosolvents to enhance the amplification process, UV irradiation of the reaction mix to avoid contamination of other DNAs, preamplification heating of the reaction mix during assembly of the components to improve product yield and specificity (D,aquila et al., 1991) and variations in the cycling times and temperatures of denaturation

including touchdown PCR where the annealing temperature is initially higher than desired and is gradually brought down to the desired temperature.

The basic PCR protocol can be manipulated in many ways to widen the variety of applications it can have. For example for site-directed mutagenesis or for introducing novel sequences onto a DNA sequence. PCR is also a quick and effective way of generating probes for other DNA manipulations. Radioactive or biotin labelling can be achieved by manufacturing primers which contain a label at the end. Radioactive probes can be made by either 5' end-labelling of the PCR primer prior to amplification, or by incorporating $^{32}P\alpha$-dCTP into the amplified sequence by including free dCTP in the amplification reaction. In general PCR plays a major role in direct and indirect mutation detection strategies negating the need for complex and time consuming cloning strategies. Several such strategies such as heteroduplex formation in heterozygotes, SSCP analysis which relies on the simultaneous labelling and amplification of gene regions, and direct sequencing which relies on a single-stranded product being produced by PCR, all rely on the amplification of specific regions and will be described in more depth. Other methods simply rely on PCR as a quick and efficient method of cloning a defined region of test DNA for further manipulation or for the generation of probes.

Several other techniques based on PCR have been developed for the analysis of mutations and particularly for mutation detection. Some of the most widely used are those strategies which rely on specific alleles being present for PCR to occur, either for correct primer annealing or for other aspects of the PCR to occur. Two such strategies, allele specific oligonulcleotide hybridisation (Saiki et al., 1986, Studencki et al., 1985) and oligo-ligation assay (Landegrin et al., 1988) are simple to perform, rapid and can be used for rapid diagnosis, but for each of these an oligonucleotide has to be synthesised which is complementary to each mutant sequence, so for conditions with heterogeneous pathologies this can be impractical and costly. For the analysis of the population frequency of common mutations however, ASO hybridisation can be a quick and effective method for quickly screening large numbers of samples.

There are disadvantages to using the PCR reaction. Primarily, accurate sequence information is a prerequisite to enable the designing of complementary oligonucleotide primers. However, methodology has been described where unknown sequences adjacent to regions of known sequence can be amplified, for example with primers directed to repetitive sequences which can pair with a primer specific to the region of interest , or inverse PCR where the target DNA is circularised before amplification . However these methods are technically difficult and have many limitations. Secondly, stringent methods are required to ensure that only the desired template is amplified and no other DNA templates are present in the reaction causing contamination during the PCR. Thirdly each primer pair has specific requirements to produce optimum results and time is often required to optimise each PCR reaction.

1.5.4 Chemical Cleavage of Mismatches (CCM)

In 1988, Cotton et al., described a method they claimed would detect all point mutations, based on the chemical reactivity of mismatched bases in DNA-DNA heteroduplexes. They described the use of reagents first used in the study of the secondary structure of tRNA and then in DNA sequencing (Maxam and Gilbert, 1980), that led to the subsequent cleavage of the DNA chain on reaction with piperidine. The chemicals shown to be most effective were osmium tetroxide and hydroxylamine which reacted with mismatched thymidines and cytidines respectively. These chemicals react specifically with pyrimidine bases in DNA. Hydroxylamine at pH 6 modifies the C5=C6 double bond in cytosine which then labilises the ring to internal rearrangement and cleavage (Rubin and Schmidd, 1980). Osmium tetroxide modification is a thymidine specific reaction in which the C5=C6 double bond is oxidised (Burtan and Riley, 1966; Friedmann and Brown, 1978,). Double-stranded DNA is attacked very slowly while single stranded regions such as at the site of mismatches are readily modified. These modifications are recognised and cleaved by piperidine.

The protocol used for CCM is outlined in figure 1.4. Reference DNA probe is mixed with excess test DNA or RNA and heteroduplexes are encouraged to form by melting the DNA and cooling at optimum temperatures. During melting and reannealing several species of duplexes are present, and these are then treated as shown in figure 1.4. After the

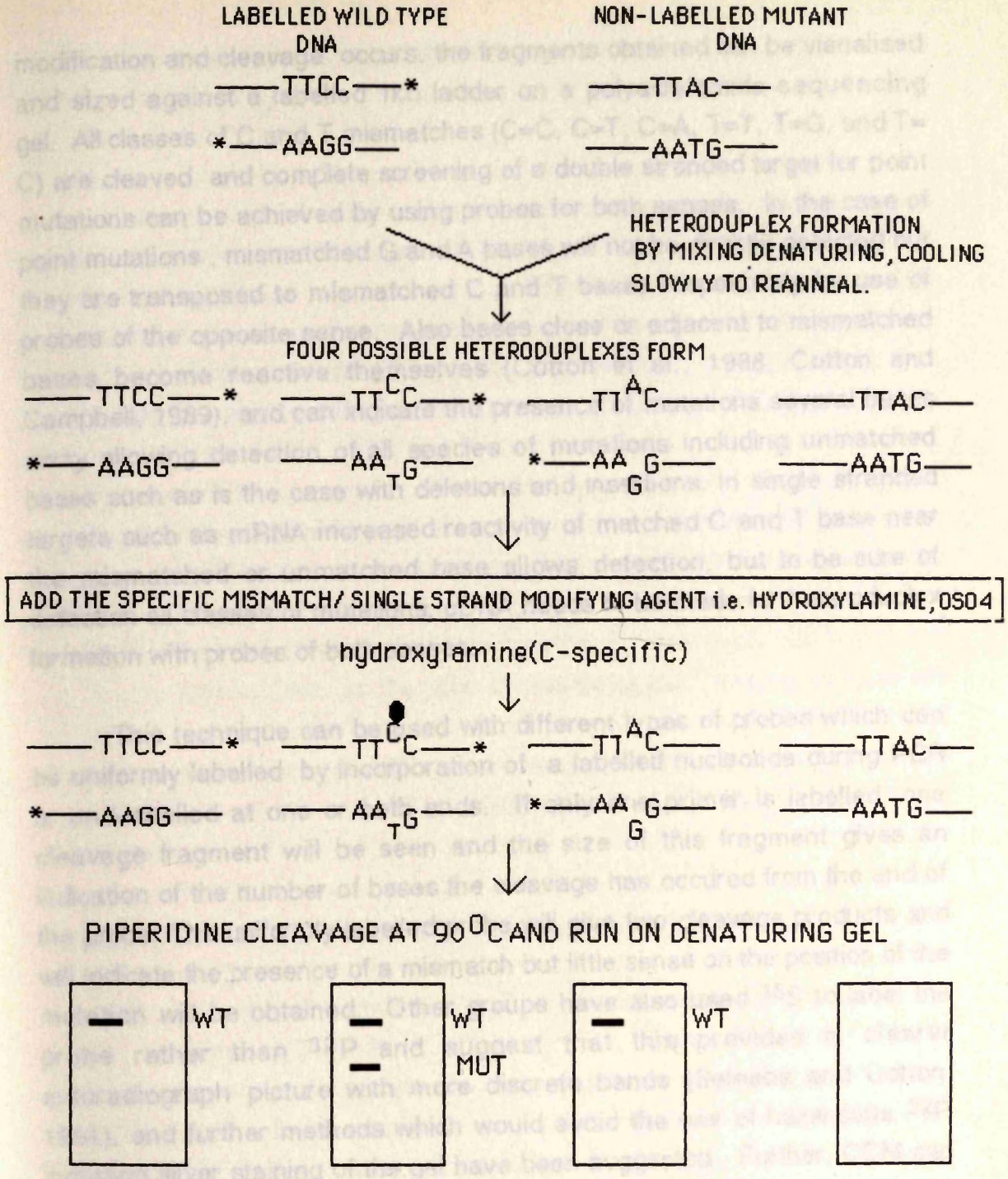


Figure 1.4. Strategy for CCM analysis. An asterisk denotes the position of the 32P at termini of end-labelled probe strands. WT= wild-type, MUT= mutant. ● = modified DNA.

modification and cleavage occurs, the fragments obtained can be visualised and sized against a labelled 1kb ladder on a polyacrylamide sequencing gel. All classes of C and T mismatches (C=C, C=T, C=A, T=T, T=G, and T=C) are cleaved and complete screening of a double stranded target for point mutations can be achieved by using probes for both senses. In the case of point mutations , mismatched G and A bases will not be directly detected but they are transposed to mismatched C and T bases respectively by use of probes of the opposite sense. Also bases close or adjacent to mismatched bases become reactive themselves (Cotton et al., 1988, Cotton and Campbell, 1989), and can indicate the presence of mutations several bases away allowing detection of all species of mutations including unmatched bases such as is the case with deletions and insertions. In single stranded targets such as mRNA increased reactivity of matched C and T base near the mismatched or unmatched base allows detection, but to be sure of detection all classes of mutations, cDNA needs to be made for heteroduplex formation with probes of both senses.

This technique can be used with different types of probes which can be uniformly labelled by incorporation of a labelled nucleotide during PCR or end-labelled at one or both ends. If only one primer is labelled, one cleavage fragment will be seen and the size of this fragment gives an indication of the number of bases the cleavage has occured from the end of the probe. The uniformly labelled probe will give two cleavage products and will indicate the presence of a mismatch but little sense on the position of the mutation will be obtained. Other groups have also used ^{35}S to label the probe rather than ^{32}P and suggest that this provides a clearer autoradiograph picture with more discrete bands (Saleeba and Cotton, 1991), and further methods which would avoid the use of hazardous ^{32}P including silver staining of the gel have been suggested . Further, CCM can be carries out using probes from the patients themselves and forming duplexes with the patients own DNA (Dianzani et al., 1991).

CCM has been successfully utilised in the study of many genetic diseases to date including haemophilia B (Montadon et al., 1989,), ornithine transcarbamylase deficiency (Grompe et al., 1989,1991), dihydropteindine reductase gene (Howells et al., 1990) and Tay-Sachs disease (Akli et al., 1991). It has been shown to detect the majority of mutations but does have the disadvantages of involving the use of highly toxic chemicals and in being

a relatively time-consuming procedure involving many stages. It would therefore not be a suitable method for quickly screening large numbers of samples.

Another method which relies on the cleavage of mismatches in duplexes is the RNase cleavage method. This involves enzymatic cleavage of RNA at single base mismatches in a RNA:DNA hybrid. It utilises RNA probes and depends on the property of many ribonucleases that they are specific for single-stranded RNA under appropriate conditions . This has been used previously for the detection of mutations in Lesch-Nyhan syndrome (Gibbs et al., 1987) but it has been reported that it is mismatch type dependent and may only detect 50% of mutations opposed to the proposed 100% detected by CCM . A similar method was proposed for cleavage of DNA:DNA duplexes using the single strand specific S1 nuclease. This, however, resulted in only a low efficiency of mismatch cleavage. More recently, several enzymes have been described which are claimed to cleave DNA at the site of mismatches. These enzymes are claimed to cleave the majority of mismatches without the time consuming use of hazardous chemicals, but to date the enzymes are not easily commercially available and definitive information is not available as to their efficiency.

1.5.5 Single-Strand Conformational Polymorphism Analysis (SSCP)

This technique , known as single strand conformation polymorphism (SSCP) analysis (Orita et al., 1989,90), was originally carried out by digestion of genomic DNA with restriction enzymes before denaturation of the DNA and electrophoresis on a neutral polyacrylamide gel. Polymorphisms in the fragments caused band shifts that were thought to be due to base changes which resulted in conformational changes in the single-stranded DNA. Under non-denaturing conditions, single-stranded DNA has a folded conformation that is stabilised by interstrand interactions. Consequently, the conformation and hence the mobility is dependant on the sequence. Subsequent publications utilised the polymerase chain reaction to radioactively label and amplify regions of the genome before SSCP analysis (Orita et al., 1989). More recently a non-isotopic method was suggested and applied to the analysis of molecular pathologies in Tay-Sachs disease (Ainsworth et al. ,1990).

The original SSCP conditions suggested the optimisation of two parameters to obtain maximum separation of the single strands,the presence and concentration of glycerol in the polyacrylamide gel and the running temperature of the gel which is usually approximately 4^0C. Although the exact role of glycerol is unknown, it has been hypothesised that its weak denaturing action on nucleic acids partially opens up the folded structure of single-stranded DNA so that more surface area of the molecule is exposed so there is more chance for the acrylamide matrix to distinguish the structural changes caused by the mutation . The percentage of acrylamide is also important as it is known that complementary single strands are better separated in gels with low cross linking . A gel with a lower percentage of crosslinking is more soft and has a larger pore size and seems to be more sensitive to conformation . As these conditions sometimes lead to incomplete separation of the strands or failure of mutations to be recognised, Spinardi et al., (1991) suggested alterations in the standard parameters by reducing the glycerol concentration and the ionic strength of the running buffer and alterations in the running conditions and temperatures. Ravnik-Glavac et al., (1994), suggested further alterations in gel matrix make up and running conditions which they suggest, further improve the detection rates, by looking for the formation of heteroduplexes as well as gel shifts on SSCP gels. These results suggested that the mobility of single stranded molecules can be affected by variations in the electrophoresis conditions and that it is possible to take advantage of these variations to optimise the protocol for different DNA segments to detect different point mutations.

Variations on the basic SSCP methodology have recently been suggested including the suggestion of two dimensional SSCP analysis (Kovar et al., 1991), to identify point mutations, deletions and insertions in long DNA fragments. Non-isotopic methods employing silver staining (Ainsworth et al. 1990), and ethidium bromide staining (Yap and McGee, 1991) have also been used. RNA-SSCP analysis has also been carried out and has ben suggested to yield better results than standard DNA-SSCP. Further, Sarkar et al., (1992), suggested a method for detection of single base changes which combines dideoxy sequencing and conventional SSCP analysis. Primarily a sequencing protocol is carried out using one of the four standard dideoxy sequencing reactions which is then run on a non-denaturing polyacrylamide gel. Depending on the nature of the sequence

change the conformation of the bands running on the gel will be altered relative to the normal sequence and a mutation can be identified. It was claimed that this protocol would detect 100% of mutations but relies on the ability to obtain good sequence data.

The standard SSCP technique although unable to give exact information as to the exact position and nature of the nucleotide change, can when optimised, indicate in which segment of the DNA or gene it is located. It is also a quick and simple and relatively inexpensive protocol which requires no specialist equipment. The effect of sequence change on electrophoretic mobility is however unpredictable and a percentage of sequence changes may not appreciably change the mobility leading to non-detection of the mutations.

All the available information would suggest that smaller sized amplified fragments are the most suitable for SSCP analysis. Orita et al. (1990), suggested that the technique can be applied to fragments up to 400 bp in size and they quote a sensitivity of 83% in 12 samples. Beck et al., (personal communication), carried out a comprehensive study of the mutation detection efficiency in a variety of fragments varying in length from 115bp to 600bp. The highest detection efficiency (97%) was detected in fragments of 155bp and only 1/29 mutations were detected in fragments of 600bp. Their final conclusion was that 80% of single base substitutions could be detected in fragments of 200bp or less. Other groups (Condie et al., 1993), suggest a greater overall detection rate of 90% in fragments of less than 400bp and different studies from different groups suggest that when a spectrum of conditions altering the electrophoresis conditions and gel make up are employed, the majority of mutations can be detected.

1.5.6 Heteroduplex Detection on Hydrolink Gels(HDHG).

In 1989, Nagamine et al., reported a PCR artefact , the formation of heteroduplexes from the products of a PCR reaction amplifying homologous loci from the candidate gene for the mouse testes determining Y-gene, *mYfin* (Nagamine et al., 1989). Extra, slower migrating bands were visualised after the resolution of the PCR product obtained from amplification of a region containing two homologous sequences later discovered to differ by an 18bp deletion. The extra bands were concluded to be heteroduplexes made up of

one strand of each of the two homologous sequences of the *mYfin* gene on the mouse Y chromosome. These heteroduplex bands were only seen when the PCR products were run on a polyacrylamide gel . The anomalous migration of these heteroduplex bands was originally postulated to be due to the secondary structure of the heteroduplexes which caused a change in migration relative to the homoduplexes, and to differences in nucleotide composition. Nagamine et al., (1989), proposed two possibilities, firstly that the secondary structure in the heteroduplexes was due to the two strands being annealed along their length with the 18bp unpaired region which was deleted in one but not the other strand, forming a bulge. The second possibility was that the strands of the heteroduplexes were annealed only 3' to the deletion with the 5' ends being unpaired. Regardless of the mechanism, Nagamine et al., (1989) suggested that such heteroduplexes produce a secondary structure that slows migration through the gel and that this migration retardation is dependant on the base composition of the heteroduplex. Triggs-Raine and Gravel (1990) however, used a double strand specific restriction endonuclease to cleave a heteroduplex formed during the PCR amplification of a region of the *HEX-A* gene which is mutated in Tay-Sachs disease, (Triggs-Raine and Gravel, 1990) to cleave the heteroduplex to completion. This indicated that the heteroduplexes were annealed along their length.

This generation of heteroduplexes will theoretically occur in any PCR reaction in which two or more homologous alleles are amplified using the same set of primers. Angliani et al., (1990) suggested that heteroduplexes form during the latter stages of PCR because the DNA concentration begins to increase to such a level that the complementary strands reanneal and outcompete the hybridisation of the primers with their template strand. Nagamine et al., (1989), suggested that this phenomena would only be of use in the detection of largish deletions or insertions but Triggs-Raine and Gravel, (1990), showed that differences as small as four base pairs could even be detected using suitable gel matrices. Single base mutations have been identified by utilising the formation of heteroduplexes that are identified by electrophoresis on a denaturing gradient gel (Sheffield et al., 1989).

Identification of heteroduplexes on non-DGGE gels has been used for detection of heterozygotes for several diseases including Tay-Sachs disease (Triggs-Raine et al., 1990) and cystic fibrosis (Rommens et al.,

1990, Angliani et al., 1990). All these studies detected the heteroduplex on polyacrylamide or Nusieve (SeaKem) agarose gels and identified heteroduplexes differing by 3 or more base pairs. Keen et al., (1991), introduced a new type of non-denaturing gel matrix, Hydrolink (AT Biochem), for the detection of single base mismatches. This protocol employed a modified PCR strategy including an extra final denaturation/renaturation step and was successfully used to pick up a point mutations in an autosomal dominant retinitus pigmentosa family (ADRP) and several point mutations causing cystic fibrosis. Since then Artlich et al., (1992) and Inglehearn at al., (1992) among others, have used the methodology successfully to screen for mutations in the rhodopsin gene in a panel of patients with ADRP. A modified Hydrolink matrix the MDE (mutation detection enhancement) gel is claimed to have an increased detection sensitivity and has been used for the identification of base changes in several diseases including Duchenne muscular dystrophy. Several modifications to the original protocols have been suggested which change both PCR conditions and gel matrixes. No studies had pinpointed the percentage of mutations that this technique could detect but initial studies suggested that it identified the majority of those analysed.

1.5.7 Allele Specific Oligonucleotide Hybridisation (ASO).

Allele Specific Oligonucleotide hybridisation (ASO)(Saiki et al., 1986), is a method for the specific detection of known nucleotide changes in a given sequence. The method relies on the differentiation of normal and mutant sequences by hybridisation of a labelled probe under stringent conditions. This technique relies on accurate sequencing information being available, in order to design a small oligo of the mutated sequence which may differ from the consensus sequence by as little as one nucleotide substitution. This oligo is labelled and hybridised under very stringent conditions to amplified regions of DNA immobilised on a membrane. The conditions of hybridisation are crucial as differential hybridisation to one sequence differing to another by such a small change requires optimisation. However once optimised mutant and normal sequence can be distinguished and many DNA samples can be immobilised and screened simultaneously, producing a quick and efficient method for screening for the presence of a known mutation in a large number of samples. ASO hybridisation is ideal for screening for common mutations in a large number of samples, but the

requirement for different oligos to be designed for each individual mutation, makes it less useful for the analysis of mutations in heterogeneous diseases where only one family harbours a particular mutation.

1.5.8 Direct Sequencing of PCR products.

Sequencing of DNA has evolved greatly since the advent of the chain-termination sequencing method (Sanger et al., 1977). This involves the synthesis of a DNA strand by a DNA polymerase *in vitro* using a single-stranded DNA template. An oligonucleotide primer is annealed to the single-stranded template and synthesis is initiated at this site and terminated by the incorporation of a nucleotide analogue,a dideoxynucleotide, that will not support continued DNA elongation. These 2'3' dideoxynucleotides (ddNTP's) do not have a 3-OH group and and it is due to this group that the base cannot form a phosphodiester bond with the next incoming nucleotide. Four reactions, each with a different nucleotide take place, and the products of these reactions are a series of incompletely elongated segments . Mixes of dNTP's and one of the four ddNTP's, cause enzyme-catalysed polymerisation to be terminated in a proportion of population of chains at each site where the specific ddNTP can be incorporated. To visualise the sequence information a radioactive nucleotide is incorporated in the synthesis so the collection of chains of varying lengths can be visualised on a denaturing polyacrylamide sequencing gel.

To work optimally, the chain termination method requires a single-stranded DNA template for the subsequent strand synthesis, but double stranded DNA that has been denatured by heat or alkali can also theoretically be used. This can be achieved by traditional cloning methods such as by use of a M13 cloning vector (Messing, 1983), or by other more recent PCR based strategies. By using M13 cloning, a good yield of pure single-stranded DNA is obtained and most sequencing kits provide primers for use with these vectors. The methodology however is technically time consuming and if a large number of samples are to be sequenced, clones must be obtained for each. The advent of PCR technology has provided an alternative for obtaining single-stranded DNA. One such strategy, asymmetric PCR (Gyllensten et al., 1988), involves a two step PCR protocol where a conventional PCR is initially carried out using two primers flanking the region of interest. The double stranded product is then used as

a template for a second amplification using one of the primers in vast excess of the other limiting primer in the hope of obtaining a single-stranded DNA as the limiting primer is present in very small concentrations and is used up very quickly. The product can be obtained very quickly and can be easily cleaned to the standard required for sequencing and the whole protocol can be completed in a much shorter time than with M13 cloning . Many factors determine the quality and quantity of sequence obtained . The type of radioactivity used is an important consideration. Initially, high energy ^{32}P was used but by using the lower energy ^{35}S the technique is safer and the resolution was increased (Biggin et al., 1983). The DNA template has to be of sufficient quality and quantity to yield a good sequence and the type and quality of the DNA polymerase is also important. Ideally this would result in equal numbers of chains terminating at each nucleotide with no false termination resulting in uniform intensities of bands with no background bands.

There are a number of polymerases that can be used in dideoxy sequencing . Each of these have different properties and the polymerase used can affect the sequence obtained. Sanger originally used the Klenow fragment which had a low processivity (the average number of nucleotides synthesised before the polymerase dissociates from the template) causing a high rate of random terminations and limiting the length of DNA that could be sequenced. The best enzymes to use are the Sequenases and Sequenase 2.0 (USB) has been genetically engineered to have no 3'-5' exonuclease activity, a high procesivity and efficient use of nucleotide analogues. Sequenases are therefore the enzymes of choice for determining the sequences of long tracts of DNA. As an alternative, Taq polymerase is useful for determining the sequence of single-stranded DNA templates that form extensive secondary structures at 37^0C. As it is stable and works efficiently at $70\text{-}75^0C$ this temperature precludes the formation of these structures in the template which lead to unreadable and unsatisfactory sequences.

Kits are now commercially available for sequencing and these kits contain a variety of reagents which can be used to optimise the reactions when problems are encountered. One such artefact that can occur, compressions, arise when regions of DNA with diad symmetry containing dG and dC residues are not fully denatured during electrophoresis causing

anomalous patterns of migration in which bands of DNA become compressed to the point where they are difficult to read. Compressions are dependent on the secondary structure of the DNA and can be resolved by using a nucleotide analogue for dGTP(dITP or 7-deaza-dGTP) which pairs weakly with conventional bases and are good substrates for DNA polymerases. These base analogues when used in sequencing reactions can eliminate most compressions. Other reagents are also available which can increase the effectiveness of the polymerase and others which can optimise the sequence produced in regions of the DNA close to the sequencing primer.

As well as chemical modification the gel running conditions can also be altered to provide the maximum sequence data. The nature and concentration of the sequencing primer is also thought to be a consideration. Once optimised, this method of sequencing can provide sequence data very quickly but the level of success has been reported to be very sequence and primer dependant. Other protocols have been suggested for obtaining single-stranded DNA, including methods which produce a double-stranded product where one strand can then be separated from the other. This can be done by digestion of a phosphorylated primer which has been used for amplification of one of the strands of the product or by use of columns which retain one strand due to a modified primer. The **best** of these protocols appears to be the use of one primer which has a magnetic bead attached which can then be retained on a magnetic column. However many of these approaches are technically difficult, time consuming or expensive as is the case with the magnetic sequencing strategy.

1.5.9 Automated Fluorescent Sequencing.

Automated fluorescent sequencing is a rapidly developing technology for the sequencing of DNA templates, including double-stranded PCR products, plasmids and single-stranded templates. The automated DNA sequencer allows the interpretation of fluorescent labelled sequences using a variety of sequencing chemistries.

The most commonly employed technique for determining DNA sequence is the chain termination sequencing chemistry previously described , utilising the ability of DNA polymerase to synthesise , *in vitro* a

complementary copy of a DNA strand. In 1985 Smith et al., described a method negating the need for the use of radiochemicals. This method involved the attachment of synthetic fluorescent dyes to the 5'-termini of synthetic oligonucleotides and sequencing chemistries and apparatus were subsequently adapted and developed (Smith et al., 1986 ; Connell et al., 1987).

The fluorescent dyes can be attached to the dideoxynucleotides or the primers and both systems have their merits and drawbacks. Sequencing can be carried out using single or double-stranded DNA templates. However a different approach utilising PCR in conjunction with a sequencing protocol can be used to allow easy sequencing of PCR products, without the drawbacks of obtaining a single-stranded template. The cycle sequencing protocol using labelled dye terminators offers a convenient approach to fluorescent sequencing of double-stranded PCR products and would be useful in this study to directly sequence amplified exons from the genes of interest.

In this sequencing chemistry, extension products are labelled by the incorporation of dideoxynucleoside triphosphates with the fluorescent dyes attached using Taq Polymerase. *In vitro* DNA synthesis requires a single-stranded template and during cycle sequencing, DNA molecules are denatured using heat to break the hydrogen bonds and then primer annealing occurs when the temperature is brought down below the primer melting temperature. By using a thermostable polymerase, extension can occur and the process can be repeated continuously with repeated cycles of denaturation, annealing and extension. The fluorescent signal can then be amplified to levels which allow enhanced sequencing data to be obtained and interpreted by suitable software. This offers an alternative to conventional sequencing and may allow areas which are problematic for conventional methodology due to difficulty in obtaining a single-stranded product to be sequenced.

1.6 Aims of the Project.

The overall aim of the present study was to optimise and compare several PCR based mutation detection strategies by using the multi-exonic *HGPRT*, *DMD* and *BRCA1* genes as model genes. The specific targets are summarised as follows:

1) To optimise several mutation detection strategies including SSCP, chemical cleavage of mismatches, heteroduplex detection using hydrolink gels, PCR based linkage analysis and different sequencing strategies, using a panel of 10 previously characterised *HGPRT* pathologies.

2) To apply the mutation detection protocol to the identification of mutations in individuals with HGPRT deficiency.

3) To use the technology established coupled with linkage analysis using a tetranucleotide repeat located within the *HGPRT* gene to designate carrier status to as many females as possible from families with an affected boy with Lesch-Nyhan syndrome.

4) To use the optimised protocols to look for small mutations in boys with DMD/BMD where the presence of a large deletion had previously been excluded.

5) To use the optimised protocols to identify mutations and polymorphisms in the *BRCA1* gene, in families with multiple breast/ovarian cancer cases.

CHAPTER 2

MATERIALS AND METHODS

2.1 Patients.

2.1.1 HGPRT Deficiency.

The patients in this study had clinically designated Lesch-Nyhan syndrome or HGPRT associated gout. Some of the index cases and family members were referred to the Duncan Guthrie Institute of Medical Genetics for biochemical diagnosis and DNA analysis or for confirmation of a biochemical analysis carried out elsewhere. The remainder of the families were originally given to us by Dr Dorothy Gibbs as frozen blood or lymphoblastoid and fibroblast cell lines.

2.1.2 Duchenne/Becker Muscular Dystrophy.

The 14 patients (DMD and BMD) in this study, were referred to the Duncan Guthrie Institute of Medical Genetics which as part of the Scottish Molecular Genetics Consortium is responsible for prenatal and carrier detection of X-linked muscular dystrophies. Each case has been clinically assessed with muscle histology undertaken and DMD and BMD differentiated. The patients chosen showed no large exon deletions with Southern blotting or PCR analysis.

2.1.3 BRCA1 Families.

DNA samples were supplied by several sources. Familial and sporadic cancer DNA samples extracted from blood, were supplied from familial cancer clinics in Glasgow and from other clinics in Scotland via Professor Michael Steel. Sporadic ovarian cancer blood samples were obtained from the Beatson Oncology unit at the Western infirmary in Glasgow and DNA was extracted as described below.

2.2 DNA Extraction from Blood.

DNA was isolated from blood based on the protocol described by Miller et al., (1988). The blood samples were thawed , transferred to a falcon

tube and lysis buffer (0.32 M sucrose, 10 mM Tris pH 7.5 , 5 mM $MgCl_2$,1% Triton x 100) added to fill up the tube. After mixing the samples were spun at 2.8 K for 10 min, the top layer discarded and the pellet resuspended in 3 ml nuclei lysis mix (10 mM Tris, 0.4 M NaCl, 2 mM EDTA pH 8.2) . 200 µl 10% SDS and 100 µl Proteinase K were added and the mixture incubated at 55^0C for 4h. 1ml of 6M NaCl was added and the sample shaken vigorously and spun at 2.5 K for 15 min. The top layer was removed to a fresh tube and 2 volumes of phenol /chloroform added before spinning for 15 min. The top layer was then removed and the previous step repeated using again phenol/chloroform and then once again using chloroform alone to remove any remaining phenol. The DNA was precipitated by the addition of 30 µl 3M Na acetate and 2 volumes of 100% ethanol and was then spooled-out using a sealed pasteur pipette , air dried and resuspended in 500µl 1x T.E. buffer. The concentration of DNA was calculated by measuring the optical density at 260 nm and the DNA stored at 4^0C.

2.3 DNA Extraction from Lymphoblastoid and Fibroblast Cell Lines.

The frozen cell samples were defrosted on ice before the cells were pelleted by spinning at 2K for 10 minutes. The medium was removed and the cell pellet washed X2 with chorion buffer (50mM Tris pH 7.5, 100mM NaCl, 1mM EDTA). After the final spin all the liquid was removed and the cell pellet resuspended in 500 µl of chorion buffer. 20% SDS (2.5µl/100µl) and Proteinase K(1µl/100µl), were added and the sample mixed well but gently before incubation at 37^0C for 3 hours until the tissue fragment disappears. The DNA was then phenol extracted, precipitated and resuspended as described for DNA extracted from blood.

2.4 Agarose Gel Electrophoresis.

A 1 % agarose gel was prepared by dissolving 0.5 g agarose powder (SEA KEM GTG,FMC Bioproducts) in 50 ml 1x TBE buffer . The mixture was boiled ,cooled and before casting ethidium bromide added. After setting, samples were mixed with 1/3 volume of loading buffer(0.25% bromophenolblue, 0.25% xylene cyanol , 40% (w/v) sucrose in water), loaded and the gel subjected to electrophoresis at 100 mA for 45 min. The DNA was then visualised using a standard U.V. transilluminator and photographed using a polaroid camera fitted with a red filter.

2.5 Amplification of Single Exons of the *HGPRT* Gene by PCR.

For the amplification of single exons of the *HGPRT* gene, the primers sequences used are shown in Table 2.1 . Each PCR reaction used 500ng of extracted genomic DNA as a template in a reaction mix containing 50ng of the relevant sense and anti-sense primer, in a total volume of 50µl containing 250µM of each of the four deoxynucleotides and 2.5 units of Taq DNA polymerase (Boehringer), in 5µl of the 10X buffer specified by the manufacturer. The reactions were mixed and overlaid with mineral oil (Sigma).

The samples were subjected to 28 cycles of denaturation at 94^{0}C for 1 min, annealing at 61^{0}C for 1 min and extension at 68^{0}C for 1 min 30 secs. A final extension step of 68^{0}C for 7 mins was then carried out before the products were checked by electrophoresis on a 1% agarose gel. The PCR's were carried out on a Techne PHC-3 thermocycler.

2.6 Amplification of Single Exons of the *DMD* Gene by PCR.

Amplification of individual exons of the *DMD* gene was carried out using primer sets described by Chamberlain et al., (1988, 1990), Beggs et al., (1990) and Abbs et al., (1991) . Their sequence and the sizes of the amplified fragments are shown in Table 2.2a and 2.2b. The PCR reaction for amplification of exons designated a, b,c,d,f,g,h and i were carried out according to the protocol suggested by Kataki, (1992). A 50µl reaction mix was set up containing 30pm of each of the relevant sense and anti-sense primers, 500ng of genomic DNA , 8µl of dNTP mix containing 1.25mM of each nucleotide and 5µl of 10x PCR reaction buffer (Boeringer) and 2.5 units of Taq polymerase (Boeringer). The PCR reaction mix was mixed and overlaid with mineral oil (Sigma), before 31 cycles of amplification were carried out. The following cycling parameters were used: With primer sets i, c and d : 94^{0}C for 30 seconds, 55^{0}C for 30 seconds and 73^{0}C for 50 seconds with a final extension at 73^{0}C for 7 minutes. For primer set a a,b,e,g and h : 94^{0}C for 60 seconds, 55^{0}C for 60 seconds, 73^{0}C for 120 seconds and a final extension at 73^{0}C for 7 minutes. For primer set f 94^{0}C for 30 seconds, 53^{0}C for 30 seconds and 65^{0}C for 55 seconds with a final extension at 65^{0}C for 7 minutes.

TABLE 2.1. The primer sequences used for the amplification of individual exons of the *HGPRT* gene and the sequence of sequencing primers used. The primers are named after the exon they amplify. The sizes of the amplified fragments are shown. The sequence of the primers are shown in 5'-3' orientation, where the sense primer hybridises 5' of the exon and the antisense primer hybridises 3' of the exon. The references for the primer sequences are given with A: indicating Gibbs et al., (1990), B : indicating Marcus et al., (1992) and C : indicating primers designed by Boyd.

EXON	SIZE	SENSE PRIMER	ANTISENSE PRIMER	REFERENCE
1	626	TGGGACGTCTGGTCCAAGG ATTCA	CCGAACCCGGGAAACTGGC CGCCC	A
2	572	TGGGATTACACGTGTGAAC CAACC	GACTCTGGCTAGAGTTCCT TCTTC	A
3	1059	CCTTATGAAACATGAGGCC AAAGG	TGTGACACAGGCAGACTGT GGATC	A
4	334	TAGCTAGCTAACTTCTCAA ATCTTCTAG	ATTAACCTAGACTGCTTCC AAGGG	A
5	707	CAGGCTTCCAAATCCCAGC AGATG	GGGAACCACATTTTGAGAA CCACT	A
6	441	GACAGTATTGCAGTTATAC ATGGGG	CCAAAATCCTCTGCCATGC TATTC	A
7/8	1533	GATCGCTAGAGCCCAAGAA GTCAAG	TATGAGGTGCTGGAAGGAG AAAAC	A
7/8	433	CCCTGTAGTCTCTCTGTATG	TTATGAGGTGCTGGAGGAG	B
7	272	AAGTGACCATGGTACACTC AGCA	TGTGCCTCTCTACAAATAT TCTCT	C
8	314	TTGTAGAGAGGCACATTTG CCAGT	TTCTAAGGACGTCTGTACT AGACT	C
9	1278	GAGGCAGAAGTCCCATGGA TGTGT	CCGCCCAAAGGGAACTGAT AGTC	A
2 SEQ		TCTCTGATAGACTAAGGTT A	ACTCAGAACAGCTGCTGAT GT	C
3 SEQ		CACAATCACAGTTCACTC	AACCTACTGTTGCCACTAA	C
4 SEQ		TCATTTAATTTGAAGTTTG TGTGT	TACTATGTAAATTATCTCT TAGTG	C
6 SEQ		TATATTGTGACTCTGAATT	TCTTAAGTCTTATATGAGA TT	C

EXON	SIZE	SENSE PRIMER	ANTISENSE PRIMER	REFERENCE
a: exon 8	360 bp	GTCCTTTACACACTTTACC TGTTGAG	GGCCTCATTCTCATGTTCT AATTAG	A
b: exon 17	416 bp	GACTTTCGATGTTGAGATT ACTTTCCC	AAGCTTGAGATGCTCTCAC CTTTTCC	A
c: exon19	459 bp	TTCTACCACATCCCATTTC TTCCA	GATGGCAAAAGTGTTGAGA AAAAGTC	A
d: exon 44	268 bp	CTTGATCCATATGCTTTTA CCTGCA	TCCATCACCCTTCAGAACC TGATTCT	A
e: exon 45	547 bp	AAACATGGAACATCCTTGTG GGGAC	CATTCCTATTAGATCTGTC GCCCTAC	A
f: exon 48	506 bp	TTGAATACATTGGTTAAAT CCCAACATG	CCTGAATAAAGTCTTCCTT ACCACAC	A
g: exon 12	331 bp	GATAGTGGGCTTTACTTAC ATCCTTC	GAAAGCCAACATAAGATAC ACCT	B
h: exon 51	388 bp	GAAATTGGCTCTTTAGCTT GTGTTTC	GGAGAGTAAAGTGATTGGT GGAAAATC	B
i: exon 4	196bp	TTGTCGGTCTCTCTGCTGGT CAGTG	CAAAGCCCTCACTCAAACA TGAAGC	B

TABLE 2.2.a : The primer sequences used for the amplification of individual exons of the *DMD* gene. Letters a-h are the designated names of the amplified exons. The sizes of the amplified fragments are shown. The sequence of the primers are shown in 5'-3' orientation, where the sense primer hybridises 5' of the exon and the antisense primer hybridises 3' of the exon. The references for the primer sequences are given with A: indicating Chamberlain et al., (1988), and B : indicating Chamberlain et al., (1990)

TABLE 2.2.b. The primer sequences used for the amplification of individual exons of the *DMD* gene. The primers are named by the exon they amplify. Pm amplifies the muscle specific promoter plus a quarter of exon 1. The sizes of the amplified fragments are shown. The sequence of the primers are shown in 5'-3' orientation, where the sense primer hybridises 5' of the exon and the antisense primer hybridises 3' of the exon. The references for the primer sequences are given with C: indicating Beggs et al., (1990), and D : indicating Abbs et al., (1991).

EXON	SIZE	SENSE PRIMER	ANTISENSE PRIMER	REFERENCE
Pm	535	GAAGATCTAGACAGTGGAT ACATAACAAATGCATG	TTCTCCGAAGGTAATTGCC TCCCAGATCTGAGTCC	C
3	410	TCATCCATCATCTTCGGCA GATTAA	CAGGCGGTAGAGTATGCCA AATGAAAATCA	C
6	202	CCACATGTAGGTCAAAAAT GTAATGAA	GTCTCAGTAATCTTCTTAC CTATGACTATGG	C
13	238	AATAGGAGTACCTGAGATG TAGCAGAAAT	CTGACCTTAAGTTGTTCTT CCAAAGCAG	C
42	155	CACACTGTCCGTGAAGAAA CGATGATG	TTAGCACAGAGGTCAGGAG CATTGAG	D
43	357	GAACATGTCAAAGTCACTG GACTTCATGG	ATATATGTGTTACCTACCC TTGTCGGTCC	C
44	426	GTTGTGTGTACATGCTAGG TGTGTA	TCCATCACCCTTCAGAACC TGATCT	D
47	181	CGTTGTTGCATTTGTCTGTT TCAGTTAC	GTCTAACCTTTATCCACTG GAGATTTG	C
50	271	CACCAAATGGATTAAGATG TTCATGAAT	TCTCTCTCACCCAGTCATC ACTTCATAG	C
52	113	AATGCAGGATTTGGAACAG AGGCGTCC	TTCGATCCGTAATGATTGT TCTAGCCTC	C
53	212	TTGAAAGAATTCAGAATCA GTGGGATG	CTTGGTTTCTGTGATTTTCT TTTGGATTG	D
60	139	AGGAGAAATTGCGCCTCTG AAAGAGAACG	CTGCAGAAGCTTCCATCTG GTGTTCAGG	C

For amplification of the remaining exons using primer sets Pm,3,6,13,42,43,44,47,50,52,53 and 60, 50pm of each primer were used and the cycling parameters were the same as for exons a,b,e,g and h.

2.7 Amplification of Single Exons of the *BRCA1* Gene by PCR.

For the amplification of single exons of the *BRCA1* gene the primer sequences used are shown in **T**able 2.3.a and b. and are similar to those suggested by Friedman et al., 1994. Each PCR reaction used 500ng of extracted genomic DNA as a template, in a reaction mix containing 100ng of the relevant sense and antisense primers in a total volume of 50µl containing 20µM of each of the four deoxynucleotides, 5mM $MgCl_2$, 1X sequencing buffer specified by the manufacturer (Gibco BRL/ Life Technologies) and 2.5 units of Taq polymerase (Gibco BRL/ Life Technologies). The reactions were mixed and overlaid with mineral oil .

The exons were amplified using a touchdown PCR involving; 4 cycles of denaturation at 95^{0}C for 10sec, annealing at 68^{0}C for 10 secs and extension at 72^{0}C for 10secs, 4 cycles of denaturation at 95^{0}C for 10sec, annealing at 66^{0}C for 10secs and extension at 72^{0}C for 10secs, 4 cycles of denaturation at 95^{0}C for 10sec, annealing at 64^{0}C for 10secs and extension at 72^{0}C for 10 secs, 4 cycles of denaturation at 95^{0}C for 10sec, annealing at 62^{0}C for 10secs and extension at 72^{0}C for 10secs. This was followed by 30 cycles of denaturation at 95^{0}C for 10sec, annealing at 60^{0}C for 10secs and extension at 72^{0}C for 10secs. The PCR reactions were carried out on a PTC-100 Programmable Thermal Controller (MJ Research Inc.).

2.8 Amplification of cDNA from the *HPRT* Gene.

For the amplification of cDNA for the *HGPRT* gene the primers 581 (CTCTGCTCCGCCACCGGCTTCCT) and 365 (CCGCCCAAAGGGAACTGA TAGTC) were used(Jolly et al., 1983). cDNA was prepared and provided by Yorkhill DNA diagnostic service and the internal primers described above were used to amplify the region of interest. The same PCR conditions were used as for the amplification of the individual exons reaction using 500ng of total cDNA as a template.

TABLE 2.3.a. The primer sequences used for the amplification of individual exons of the *BRCA1* gene . The primers are named after the exon they amplify. The sizes of the amplified fragments are shown. The sequence of the primers are shown in 5'-3' orientation, where the sense primer hybridises 5' of the exon and the antisense primer hybridises 3' of the exon. The references for the primer sequences are given with B the sequences given by Miriad Genetics (Utah) and Friedman et al., 1994, .

EXON	SIZE	SENSE PRIMER	ANTISENSE PRIMER	REFERENCE
2	250	GAAGTTGTCATTTTATAAA CCTTT	TGTCTTTTCTTCCCTAGTAT GT	B
3	300	TCCTGACACAGCAGACATT TA	TTGGATTTTCGTTCTCACTT A	B
4	200	GTCAAAGAGATAGAATGTG AGC	CCCGTCTCTACAGAAAACA C	B
5	200	CTCTTAAGGGCAGTTGTGA G	TTCCTACTGTGGTTGCTTCC	B
6/7	420	CTTATTTTAGTGTCCTTAA AAGG	TCGGGTTCACTCTGTAGAA G	B
8	220	TGTTAGCTGACTGATGATGG T	ATCCAGCAATTATTATTAA ATAC	B
9	200	CCACAGTAGATGCTCAGTA AATA	TAGGAAAATACCAGCTTCA TAGA	B
10	220	TGGTCAGCTTTCTGTAATC G	GTATCTACCCACTCTCTTC TTCAG	B
12	220	GTCCTGCCAATGAGAAGAA A	TGTCAGCAAACCTAAGAAT GT	B
13	280	AATGGAAAGCTTCTCAAAG TA	ATGTTGGAGCTAGGTCCTT AC	B
14	250	CTAACCTGAATTATCACTA TCA	GTGTATAAATGCCTGTATG CA	B
15	250	TGGCTGCCCAGGAAGTATG	AACCAGAATATCTTTATGT AGGA	B
16	375	AATTCTTAACAGAGACCAG AAC	AAAACTCTTTCCAGAATGT TGT	B

17	350	GTGTAGAACGTGCAGGATTG	TAGCCTCATGTGGTTTTA	B
18	350	GGCTCTTTAGCTTCTTAGGAC	GAGACCATTTTCCCAGCATC	B
19	220	CTGTCATTCTTCCTGTGCTC	CATTGTTAAGGAAAGTGGTGC	B
20	220	ATATGACGTGTCTGCTCCAC	GGGAATCCAAATTACACAGC	B
21	275	AAGCTCTTCCTTTTTGAAAGTC	GTAGAGAAATAGAATAGCCTCT	B
22	275	TCCCATTGAGAGGTCTTGCT	GAGAAGACTTCTGAGGCTAC	B
23	250	CAGAGCAAGACCCTGTCTC	ACTGTGCTACTCAAGCACCA	B
24	275	ATGAATTGACACTAATCTCTGC	GTAGCCAGGACAGTAGAAGGA	B

TABLE 2.3.b. The primer sequences used for the amplification of overlapping segments of exon 11 of the *BRCA1* gene. The sizes of the amplified fragments are shown. The sequence of the primers are given in 5'-3' orientation, where the sense primer hybridises 5' of the exon and the antisense primer hybridises 3' of the exon. The references for the primer sequences are given with A indicating Friedman et al., 1994.

k11a	309	AACACCACTGAGAAGCGTG CAG	CTCACACAGGGGATCAGCA TTC	A
k11b	300	CAACATAACAGATGGGCTG GAAG	ACGTCCAATACATCAGCTA CTTTGG	A
k11c	295	GGTTCTGATGACTCACATG ATGGG	TCTGTGGCTCAGTAACAAA TGCTC	A
k11d	252	GAAAACCTATCGGAAGAAG GCAAG	TCATCACTTGACCATTCTG CTCC	A
k11e	269	ATCAGGGAACTAACCAAAC GGAG	CGCATGAATATGCCTGGTA GAAG	A
k11f	273	AGGCTGAGGAGGAAGTCTT CTACC	CAGCTCTGGGAAAGTATCG CTG	A
k11g	319	GCAACTGGAGCCAAGAAGA GTAAC	CCTGAGTGCCATAATCAGT ACCAGG	A
k11h	286	AAGTGTCTAATAATGCTGA AGACCCC	CCCAATGGATACTTAAAGC CTTCTG	A
k11i	280	GCACTCTAGGGAAGGCAAA AACAG	CATTCCTCTTCTGCATTTCC TGG	A
k11j	288	GCCAGTCATTTGCTCCGTTT TC	CGTTGCCTCTGAACTGAGA TGATAG	A
k11k	305	TGCAGGCTTTCCTGTGGTT	GGCTAATTGTGCTCACTGT ACTTGG	A
k11l	270	TCAATGTCACCTGAAAGAG AAATGG	CAGGATGCTTACAATTACT TCCAGG	A
k11m	270	TTGAATGCTATGCTTAGAT TAGGGG	GACGCTTTTGCTAAAAACA GCAG	A
k11n	253	GTTTGTTCTGAGACACCTG ATGACC	AGTGTTGGAAGCAGGGAAG CTC	A
k11o	289	GAGTCCTAGCCCTTTCACC CATAC	GTGATGTTCCTGAGATGCC TTTG	A
k11p	314	CGTTGCTACCGAGTGTCTGT CTAAG	AGCCCGTTCCTCTTTCTTCA TC	A

2.9. Visualisation of PCR Products.

10% of the PCR product was mixed with 1/3 volume of loading buffer and and the products run in a 1% agarose mini gel as described in 2.4 with the products being sized against a 1kb ladder (BRL) .

2.10 Manufacture, Preparation and Purification of Oligonucleotide Primers.

A number of oligonucleotide primers were manufactured 'in house' on an Applied Biosystems (ABI) PCR mate oligonucleotide primer synthesiser. For this study the primers were initially synthesised with the trityl group off. The primers were cleaved from the support by passing 1ml of concentrated ammonium hydroxide through the column containing the oligo in three stages, passing 330µl through the column every 20 minutes , collecting the ammonium hydroxide in a tube. The volume of ammonium hydroxide containing the cleaved primer was then increased to 2ml and the oligo deprotected by incubation at 55^0C overnight. The primers were then left open in a fumehood for 12 hours to allow the remaining ammonia to vapourise and the oligonucleotide primers then precipitated from the mix by salt/ethanol precipitation, washed with 70% ethanol and resuspended in a suitable volume of ddH2O. The concentration of the oligonucleotide was then determined by finding the O.D. at 260nm.

Due to the sensitivity of several of the amplification reactions for the *HGPRT* gene, many of the primers were manufactured with the trityl group on, to allow further purification of the primer through a OPC (oligonucleotide purification cartridge) column (ABI). After completion of the trityl-on synthesis, the oligo was cleaved from the support and deprotected as above. An OPC cartridge was then washed by flushing with 5ml HPLC grade acetonitryl followed by 5ml of 2.0M triethylamine acetate. An aliquot of a final volume of 1-4 ml containing approximately 20 O.D. units of the deprotected oligo diluted with one third volume of deionized water was then taken into a polypropylene syringe and slowly pushed through the cartridge, the eluted fraction collected and pushed through he cartridge again. The cartridge was again washed with 3X5ml of 1.5M ammonium hydroxide and 2X5ml deionised water.

The OPC bound oligo was then detritylated by addition to the column of 5ml of 2% triflouroacetic acid solution where 1ml was primarily pushed through the column and left for 5 minutes before the remainder was pushed through. The cartridge was again washed by flushing with 2X5ml deionised water before the purified, detritylated oligonucleotide was eluted by slowly washing the cartridge with 1ml of 20% acetonitryl solution. The O.D. at 260nm was then determined.

2.11 Cleaning of Genomic DNA and Oligonucleotide Primers.

Genomic DNA and oligonucleotide primers were cleaned by ethanol/salt precipitation. An aliquot of the DNA or primer was mixed with 1/10 volume of 3M sodium acetate (pH 5.5) and 2.5 X total volume of filtered 100% ethanol. The DNA was precipitated at -70^{0}C for one hour before the pellet was obtained by centrifugation at top speed in a microcentrifuge for 15 minutes. The DNA pellet was then washed twice with 70% ethanol and air dried for 15 minutes before the DNA was resuspended in an appropriate volume of T.E. buffer. The oligonucleotide primers were resuspended in ddH2O and the concentration of each determined by finding the O.D. at 260nm.

2.12 Linkage Analysis Using a Tetranucleotide Repeat Located Within the *HGPRT* Gene.

The STR within the *HGPRT* gene was amplified using flanking primers with the sequences; 5'-ATG CCA CAG ATA ATA CAC ATC CCC-3' and 5'- CTCTCCAGAATAGTTAGATGTAGG-3'. PCR was carried out in a total volume of 50µl containing 5µl of PCR buffer 200µM of each of the four dNTP's, 500ng of genomic DNA and 35pm of each purified primer one of which had been end labelled with ^{32}P γ-ATP using T4 polynucleotide kinase (NBL). After addition of 2.5 units of Taq polymerase amplification was performed with an initial denaturation of 94^{0}C for 5 min followed by 25 cycles of denaturation at 94^{0}C for 2 min, annealing at 60^{0}C for 1 min and extension at 72^{0}C for 2 min. At the end of the PCR reaction , 2 units of the Klenow fragment of DNA polymerase (BRL) was added to each sample, and incubation was continued at 37^{0}C for 15 min. 2µl of the final product was then mixed with 6µl of a formamide loading buffer and electrophoresed on a 4% polyacrylamide /7M urea sequencing gel for 4-8 hours depending on the

degree of separation required. The gel was dried and subjected to autoradiography for the required time period.

2.13 Heteroduplex Detection Using Hydrolink Gels(HDHG).

This method was based on the protocol provided by Jeffrey Keen (personal communication), and is a modification of that described by Keen et al., (1991).

PCR amplification was carried out using the reaction mixtures previously described for the individual *HGPRT* and *DMD* exons with the modification that for analysis of male DNA, 250ng of patient DNA and 250ng of control male DNA were present in each amplification reaction mix for the X-linked genes, thus creating a potential false heterozygous state. The primers used are as described previously in tables 2.1, 2.2 and 2.3 and the panel of known *HGPRT* mutations analysed is summarised in table 2.4. For amplification of the *HGPRT* exons, an initial 2 step denaturation cycle of 94^0C for 2 minutes and 60^0C for 5 minutes was followed by 30 cycles of denaturation at 94^0C for 30 seconds and a single annealing /extension step of 60^0C for 5 minutes, followed by a final denaturing/renaturing step of 94^0C for 30 seconds and 70^0C for approximately 1-16 hours. This increased the chance of heteroduplex formation between non-homologous strands. For the *DMD* gene, the PCR conditions were as for the amplification of the individual exons with the final denaturation/renaturation step as described .

The reannealed double-stranded PCR products were then subjected to electrophoresis on hydrolink D-5000 gels to resolve heteroduplex from homoduplex fragments. The gels were cast vertically in a sandwich consisting of 2, 20x20cm or 20 X30cm plates clamped together and separated by 1.5 mm spacers and sealed at the bottom with plasticine. A mix containing 35.4 mls of Hydrolink D-5000 (AT Biochem) and 4mls 10 x TBE buffer was polymerised by addition of 25 µl N,N,N',N' tetramethylenediamine (TEMED)(Sigma) and 750 µl fresh 10 % ammonium persulphate. This was poured between the two plates, a 10 well comb inserted and the gel left to set for approximately 1 hour. After removing the comb the wells were rinsed with the running buffer (1xTBE buffer) , and 16 µl of the PCR product + 4 µl of the loading buffer (5% sucrose, 0.06% orange G) were loaded and run at different voltages for different times depending on

MUTATION	SEQUENCE CHANGE	AA CHANGE
HGRRT CODICOTE	T298[a]	P
HGPRT CHELTENHAM	GT[b] 288-289	P
HGPRT MASHAD	A59T	D20V
HGPRT SHEFFORD	C151T	R51X
HPRT BANBURY	G152C	R51P
HGPRT RUNCORN	T395C	I132T
HGPRT FARNHAM	C486G	S162R
HGPRT NORTH MYMMS	C508T	R170X
HGPRT MARLOW	C527T	P176L
HGPRT READING	G617A	C206Y

Table 2.4 Summary of the panel of known mutations in families with Lesch-Nyhan syndrome . The amino acid and nucleotide changes present are indicated. a-indicates an insertion, b-indicates a deletion, P-indicates premature termination.

the size of amplification product to be analysed (Table 3.1)(Boyd et al., (1993)).

The gel was then stained with ethidium bromide for 20 minutes followed by destaining in the running buffer for 4 hours and the DNA visualised and photographed as previously described.

2.14 Purification of PCR Products.

2.14.1 Geneclean.

Geneclean was used to purify non-radioactive PCR products of over 500 bp in size. After removal of the mineral oil the total PCR product was transferred to a 1.5 ml eppendorf tube and the product was cleaned using Geneclean 2. kit (Bio 101 inc.). Three volumes of sodium iodide solution and 5µl of glassmilk suspension were added and the sample vortexed and incubated on ice for 5 minutes, mixing every two minutes, to allow the binding of DNA to the silica matrix. The glassmilk/DNA was then pelleted and washed three times with NEW WASH(10ml dH2O, 11ml 100% ETOH, 500µl NEW concentrate). After the third wash the pellet was then dried and the DNA eluted from the glassmilk by dissolving the pellet in 20µl of T.E. buffer and incubated at 55^0C for 3 minutes. The mix was then spun at top speed in a microcentrifuge to pellet the glass milk and the supernatant containing the DNA removed to a new tube.

2.14.2 Purification of PCR Products Through an Agarose Gel.

This method was used to purify radioactive PCR products and products smaller than 500bp. After mixing with 10% volume of loading buffer, the entire PCR product was subjected to electrophoresis through a 1% agarose gel containing ethidium bromide, for 45 minutes at 100 Volts. The product was visualised on a U.V. transilluminator with a thick perspex sheet between the gel and the U.V. source to minimalise the degradation of the DNA by the U.V. light. The slice of gel containing the amplified fragment was cut from the gel and placed into a 1.5 ml autoclaved eppendorf with a hole in the bottom, and which was half filled with siliconised, autoclaved glass wool. A second eppendorf was placed under the first, both were labelled and the double eppendorf placed in a suitable centrifuge and spun

at top speed for 15 minutes or until the entire gel slice had collapsed to a liquid containing the DNA in the bottom eppendorf. The DNA was then precipitated by salt/ethanol precipitation, washed with 70% ethanol and resuspended in 200µl of ddH_2O.

2.15 Radioactive Labelling of Oligonucleotide Primers.

Oligonucleotide primers were 5'-end labelled individually with enough primer for two PCR reactions being labelled in one reaction tube. The labelling mix containing the primers, 10 units of T4 polynucleotide kinase, 1x 1 phos-all buffer(Pharmacia) and 25µci of ^{32}P γ–ATP was mixed before incubation at 37^0C for one hour before half of each reaction mix was used for each PCR reaction.

2.16 PCR-SSCP Analysis.

PCR was carried out for the individual *HGPRT*, *DMD* and *BRCA1* exons as described in 2.5, 2.6 and 2.7 either using the 5'-end labelled primers or containing 25µci of α-dCTP as described for internal labelling . The kinased primers were added without purification.

The amplified products were diluted 1:25 with 1% SDS, 10mM EDTA, followed by a 1:2 dilution in 95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% Xylene cyanol. The sample was heated to 100^0C for 10 minutes to denature the DNA and then snap cooled on ice before loading on a 6.5% non-denaturing polyacrylamide gel containing varying concentrations of glycerol and was run at different wattages for different times to obtain optimum separation of the single strands. The 6.5 % non-denaturing polyacrylamide gels were prepared as with denaturing sequencing gels as described in 2.26 with the absence of urea and glycerol added at the relevant concentration. The gels containing glycerol were run at room temperature with a fan directed at the plates to ensure the gels were cool. Gels without glycerol were run in the cold room at 4^0C. The gels were dried on a gel drier and subject to autoradiography at -70^0C overnight.

2.17 Autoradiography.

The gel was placed beside an X-ray film (Kodak Diagnostic: X-OMAT TM AR 5) in a light tight cassette containing intensifying screens. The cassette was left for the desired time in a - 70^0 C freezer and the film developed using a Fugi X-Ray film processor.

2.18 Restriction Enzyme Digestion of PCR Products.

Non-labelled PCR products were digested with the relative restriction enzymes by incubating at 37^0C for 1 hour, 15µl of PCR product with 2µl of restriction enzyme and 2µl of the correct react buffer and 1µl of ddH_2O. Exons 2 and 5 were digested with Dde 1, exon 3 with Alu 1, exon 7 with Hinf 1 . For SSCP analysis, the PCR product was digested as above and 5µl of the digested fragment was incubated at 37^0C for 1 hour with 2µl 1-phos-all buffer (Pharmacia), 3µl of γ–ATP 2 units of T4 polynucleotide kinase and the volume made to 20 µl with ddH_2O.

2.19 Chemical Cleavage of Mismatches (CCM).

To screen for mutations , the CCM method described by Cotton et al., (1988) and Dahl et al., (1989), was originally used before the modified version of the protocol as documented by Howells et al., (1990), was employed.

The nature of the chemicals used in this procedure merited caution in the handling and disposal of the solutions. The procedure was carried out in a fumehood, with the apparatus used being used only for this protocol. No other work was carried out in the fumehood while the toxic chemicals were in use and special protective clothing, facemasks, safety glasses and double gloves were used. The chemicals were disposed of only down the fumehood sink and plasticware and gloves which was in contact with the chemicals was placed in a cinbin in the hood and disposed of once full by burning. The chemicals were stored in the fumehood or in specially labelled containers in special fridges not in general use.

2.19.1 Method 1, (Cotton et al. , (1988))

The regions of the patient DNA of interest ,were amplified according to the protocols previously described in 2.5 and 2.6. The PCR products were then directly cleaned using a Geneclean II Kit (BIO 101 Inc.) if the amplified fragment was greater than 500 bp in size as described in 2.14.1. If the amplified fragment was less than 500 bp in size the fragment was purified through agarose gel as described in 2.14.2. The final cleaned DNA was either eluted or resuspended in a final volume of 20 µl of T.E. buffer.

The original probes were prepared by kinase labelling of the amplified fragment, (Maniatis et al. (1989)). 100 ng of the PCR product was labelled by addition of 1xKinase buffer(0.5M Tris-Cl(pH 7.6),0.1 M $MgCl_2$, 50 mM dithiothreitol,1mM spermidine, 1 mM EDTA,),20 µCi ^{32}P γ-ATP, 10 units of T4 polynucleotide kinase(NBL) and dH_2O to 20 µl. After mixing, the reaction was incubated at 37^0C for 30 minutes. The labelled DNA was then precipitated by addition of 2 volumes of 2M ammonium acetate and 3 volumes of 100% ethanol. After precipitation for 1 hour at -70^0C, the DNA was spun down and washed x2 with 70% ethanol before drying and resuspension in 50 µl of ddH_2O. The amount of incorporated radioactivity was calculated by measuring the counts per minute using a scintillation counter. 2 µl of a 1:2 dilution of 5µl of the labelled product was spotted onto 2 separate circles of Wattman filter paper. One of these was then washed x4 with 0.5M Na2HPo4, x2 with ddH_2O and x1 with 100 % ethanol. Both pieces were then dried placed in a polyurathane scintillation vial containing scintillation fluid and the counts per minute of the washed and unwashed DNA samples measured and compared. Only probes with more than 20% incorporation of radioactivity were used.

2-5 ng of labelled control DNA was mixed with at least 12x excess of unlabelled test DNA or unlabelled control DNA in a screw capped 1.5 ml Sarstedt vial. To this an equal volume of 2x annealing buffer(1.2M NaCl, 12mM Tris-HCl pH 7.5, 14mM $MgCl_2$) was added and the mixture boiled for 5 minutes before removal to a 42^0C to allow annealing of the strands. After 1 hour incubation the heteroduplexes were precipitated in an equal volume of 5M sodium acetate(pH 5.5)and 2.5 volumes of 100 % ethanol with 4µl of 20mg/ml glycogen (**Boeh**ringer) added to facilitate precipitation. After

spinning down, washing and drying the pellet in the usual manner, the pellet was resuspended in 12 µl ddH_2O.

6 µl of the heteroduplex mixture was added to two fresh Sarstedt tubes. To one tube 20µl of hydroxylamine solution (Aldrich) (1.39g hydroxylamine in 1.6ml ddH_20, adjusted to pH 6.0 by addition of 1ml diethylamine (Aldrich)) was added and the mixtures incubated for 60 minutes at 37^0C. 2.5µl of 10X OsO_4 buffer (10mM Tris-HCL pH 7.7, 1mM EDTA, 1.5% pyridine) and 15µl of a 1/5 dilution of OsO_4 (Aldrich) (0.5g/12.5 ml distilled water) were added to the other tube and this was incubated at 37^0C for 5 minutes. After incubation the reaction was stopped by addition of 200µl of "HOT STOP" solution (0.3M sodium acetate pH 5.2, 0.1mM EDTA, 25mg tRNA/ml), and 750µl of 100% ethanol were added and the DNA precipitated at -70^0C. After centrifugation the DNA was resuspended in 200µl of 0.3M sodium acetate pH 5.2 and 500µl 100% ethanol added and the DNA was reprecipitated, ethanol washed and dried.

50µl of 10% piperidine (Aldrich) was added to each dried pellet which was then vortexed for 10 seconds before incubation at 90^0C for 30 minutes. After cooling on ice the heteroduplexes were then precipitated at -70^0C by addition of 50µl of 0.6M sodium acetate pH 5.2 and 300µl of 100% ethanol before centrifugation and washing with 70% ethanol. After drying the pellets were resuspended in 8µl of loading buffer (0.05% bromophenol blue, 0.05% xylene cyanol FF, and 0.2 ml of 0.5mM EDTA dissolved in 10ml formamide). The samples were boiled for 10 minutes and cooled on ice before loading on an 8% denaturing polyacrylamide gel prepared as described in 2.26 with a labelled 1kb ladder . The gels were run for approximately two hours or until the bromophenol blue marker had run 30cm and the gel was then fixed and dried as described. The gel was then subjected to autoradiography at -70^0C as described for 7 days.

2.19.2 Method 2 .

This method utilised different methods of probe preparation and was based on the methodology suggested by Howells et al., (1990). Two different strategies were employed to achieve optimal labelling of the DNA probe, namely, incorporation of ^{32}P α-dCTP into the DNA during

amplification and labelling of the oliginucleotide primers by 5' end-labelling using $^{32}P\gamma$-ATP.

For incorporation of radioactivity along the whole length of the DNA probe the PCR for the individual exons was as described in 2.5 and 2.6 with the modification that 20pm of $^{32}P\ \alpha$-dCTP (60µci) were added to the reaction mix and the quantity of non-radioactice dCTP was reduced from 200µM to 6µM . To avoid misincorporation of alternative dNTPs as dCTP concentrations fell only 25 PCR cycles were performed. For an end-labelled probe, the primers were 5'-end labelled as described in 2.15 and the PCR was carried out otherwise as described in 2.5 and 2.6. The entire PCR product was run in a 1% agarose gel and the probe purified and isolated as described in 2.14.2, before resuspension in 1X T.E. (10mm Tris-HCL, 1mm EDTA, pH 7.4) at 1000cpm/ml.

The heteroduplexes and control homoduplexes were formed in siliconised Sarstedt tubes . 200 ng of test DNA (approximately 1/4 of the PCR product) and 20ng of purified probe (56,000 cpm) were added to the siliconised tubes and dd$H_2$0 added to the volume of 128µl. 128 µl of annealing buffer as described above was added and the tubes capped with punctured non-siliconised lids before boiling for 5 minutes. The tubes were then capped with non-punctured siliconised caps and the heteroduplexes were formed at 42^{0}C for 60 minutes as described. The remainder of the protocol was as described for method 1, but after the piperidine cleavage and addition of the formamide buffer the DNA was left to resuspend overnight before electrophoresis.

2.20 Radioactive Labelling of a 1kb Ladder.

The DNA fragments of the 1kb ladder were 5'-end labelled using ^{32}P γ-ATP. 3.5µl of ladder (BRL) was incubated at 37^{0}C for 1 hour with 2.5µl of $^{32}P\ \gamma$-ATP, 1µl of 10x one-phos-all buffer (Pharmacia), 10 units of T4 polynucleotide kinase (NBL) and ddH_2O to make the volume to 10µl. The labelled ladder was stored at -70^{0}C and before electrophoresis an aliquot of the ladder(depending on the age of the ladder) was mixed with 1/2 volume of formamide loading buffer and heated to 100^{0}C for 5 minutes immediately before loading.

2.21 Allele Specific Oligonucleotide Hybridisation.

PCR reaction were carried out for the exons of interest as described previously in 2.7. 20 µl of each PCR reaction was transferred to a fresh tube and the samples heated to 95^{0}C before chilling on ice. An equal volume of 20X SSC was added and the samples spotted in 5µl aliquots onto a Hybond N $^+$ membrane prewetted with 10X SSC and stamped with an ink grid. The samples were allowed to dry before the addition of the next DNA aliquot. The membrane was then denatured by placing DNA side up on a piece of filter paper soaked in deaturation solution (1.5M NaCl, 0.5M NaOH) for 5 minutes. The filter was then placed on a piece of filter paper soaked in neutralising solution (1.5M NaCl, 0.5M Tris-HCl pH 7.2, 0.001M EDTA) for 1 min. and then air dried before fixing by U.V. crosslinking.

Membranes were pre-hybridised for 2 h at 52^{0}C with 5ml of a solution containing 3.0M tetramethyl ammoniumchloride (TMAC), 0.1M NaPo4 pH 6.8, 1mM EDTA, 5X Denhardts solution, 0.6% SDS and 100µg/ml yeast tRNA. The membranes were then probed overnight at 52^{0}C by addition of all of 500ng of a γ^{32}P end labelled oligonucleotide primer , whose sequece corresponded to the mutated sequences of the AG deletion in exon 2 the C insertion in exon 20, and the AA deletion in exon 11. The sequences of these were ATC TTA GTG TCC CAT CT, GAG AAT CCC CAG GAC AG and GTC CTT AAA GAC AAA GT respectively and the primers were designed with the variant sequence in the centre of the primer sequence. After probing the membranes were washed with 2X SSC for 20 mins. at RT with agitation and then for 20 mins. at 52^{0}C in a buffer containing 3.0m TMAC, 1mM EDTA, 10mM NaPo4 and 0.6% SDS. The filters were then air dried, covered with cling film and exposed to X-Ray film at -70^{0}C for 48 hours.

2.22 Direct Sequencing of Single-Stranded DNA Templates Generated by Asymmetric PCR.

Direct sequencing of single-stranded asymmetric PCR products was carried out using a Sequenase Version 2.0 sequencing kit (United States Biochemical). Single-stranded DNA templates were prepared by carrying out a PCR reaction with the concentration of either the sense of antisense primer being in 50 - 75 x excess of the other primer. The PCR reaction mixture and conditions were otherwise the same as previously described

for PCR amplification of the individual *HGPRT* and *DMD* exons except that the reactions were carried out in a final volume of 100µl with 3.5 units of Taq polymerase and 10 more cycles of amplification were carried out. The asymmetric PCR product was checked for the presence of a potential single stranded product on a 1% agarose mini gel . The product was cleaned by precipitating the DNA at room temperature for 30 minutes with the addition of equal volumes of 4M ammonium acetate and 2.5 x total volume of 100% isopropanol before precipitation of the DNA in a microcentrifuge for 15 minutes at top speed, washing of the DNA in 70% ethanol and after drying , resuspending the pellet in 7µl of ddH_20 (Charles Mgone, personal communication).

Direct sequencing was carried out using the chain-termination DNA sequencing method (Sanger et al., 1977). This was carried out according to the manufacturers of the sequenase version 2.0 kit instructions.1 pm of the primer limited in the asymmetric PCR was added to the annealing mixture containing 7µl of single stranded DNA (the entire PCR product) and 2µl of 5X sequencing buffer before annealing was carried out by heating the mixture to 65^0C for two minutes before slow cooling to below 35^0C. The labelling reaction consisting of 1µl of 0.1M Dithiothreitol (DTT), 2µl diluted labelling mix (1.5 mM each of dGTP, dCTP and dTTP), 1000mCi/mMol of ^{35}S dATP and 3 units of Sequenase T7 DNA polymerase being incubated at room temperature for 5 minutes . 3.5µl of the labelling reaction was transferred to 2.5µl of each dideoxy termination mix prewarmed to 37^0C. The termination reaction was carried out at 37^0C for 4 minutes before the reactions were stopped by addition of 4µl of Stop solution.

Samples were heated to 75^0C for two minutes, snap cooled on ice and loaded on a 5% polyacrylamide sequencing gel. Different modifications were used in an attempt to optimise the signal including; addition of Mn Buffer which is a reagent which takes advantage of the enhanced activity of the Sequenase 2.0 enzyme in the presence of mn 2^+ions altering the concentration of primer used, increasing the concentration of DNA in the reaction and using nucleotide analogues provided in the kit to weaken the CG bonds and limit stops in the sequencing reaction and altering the extension and termination temperatures and times.

2.23 Sequencing of Double-Stranded PCR Products.

The exon of interest was amplified as normal using the PCR reaction and the double-stranded DNA product was precipitated at -70^{0}C after addition of 1/10 volume of 3M Sodium Acetate (pH5.5) and 2.5X volume of 100% ethanol. The pellet was washed with 70% ethanol and air dried. To denature the DS DNA, a final concentration of 0.2M NaOH and 0.2 mM EDTA (pH 8) was added to the DNA which was incubated at 37^{0}C for 30 minutes. The reaction was then neutralised by addition of 1/10 volume of 3M sodium acetate (pH 5.5). The samples were ethanol precipitated, washed at least twice with 70% ETOH and the pellets of DNA resuspended in 7µl of ddH_2O. The samples were thereafter treated according to the protocol provided with the Sequenase 2 kit as described in 2.22.

2.24 Sequencing with Formamide.

This was carried out according to the protocol suggested by Zhang et al., (1991). 50% of a double-stranded PCR product (10µl) was cleaned using geneclean and mixed with a five molar excess of sequencing primer in 1X sequencing buffer with 3µl of formamide. The annealing mixture was boiled for 10 minutes and cooled in a dry ice/ethanol bath. The sequencing reaction was then carried out as described in 2.22 with the exception that the sequencing reaction was carried out on ice for ten minutes and the termination reaction at 42^{0}C fro 10 minutes. Before loading on a sequencing gel the reactions were denatured at 95^{0}C for 3 minutes.

2.25 Production of a Single-Stranded DNA Template by Exonuclease Digestion.

This was carried out according to the method described by Higuchi and Ochman, (1989). 50pm of one of the oligonucleotide primers is 5'-end labelled using 2 units of T4 polynucleotide kinase and 1mm of ATP at 37^{0}C for 1 hour. The kinased primers were then precipitated in 0.3M sodium acetate, 0.01mM $MgCl_2$ and 2 volumes of 100% ethanol and the resuspended primer added to a PCR reaction mix containing an equal concentration of the opposing non-kinased primer and amplification was carried out using the normal conditions. After amplification the double-stranded product was extracted with phenol, precipitated with 3M sodium

acetate and 100% ethanol and treated at 37^{0}C for 15 minutes with 4 units of lambda exonuclease (BRL) in 50µl of a buffer containing 67mM glycine-NaOH (pH 9.4), and 2.5mM $MgCl_2$. The single-stranded product was extracted twice with phenol, once with phenol/chloroform and ethanol precipitated as previously described. The single stranded template was then sequenced as usual using the Sequenase 2.0 kit (USB)(2.22) and the sequence visualised on a denaturing sequencing gel.

2.26 Preparation of Sequencing Gels.

8% denaturing sequencing gels were prepared in a vertical gel cast as follows. The glass plates were washed thoroughly with decon, rinsed with distilled water and cleaned with 70% ethanol. 0.4mm spacers were placed along the edge of one of the glass plates and the plates clamped together. The bottom edge of the plates were sealed and a mix containing 12 mls 40 % acrylamide (40 % (w/v) acrylamide <19;1> bisacrylamide solution, (NBL)), 6ml 10X TBE buffer, 42ml dH_2O and 25g urea(7M) was polymerised by addition of 100 µl each of 20 % ammonium persulphate and TEMED and carefully poured down the edge avoiding the formation of air bubbles.

A comb was placed at the top edge of the gel which was left to set for one hour. The comb was carefully removed and the wells cleaned thoroughly by flushing with the running buffer to remove any urea and excess acrylamide.

2.27 Electrophoresis of Denaturing Sequencing Gels.

Sequencing gels were run at approximately 2000 volts and before loading of the samples, gels were preheated to 50^{0}C. After running for variable amounts of time depending on which part of the sequence was required , the gel was fixed in 10% methanol, 10 % acetic acid for 15 minutes and dried for 30 minutes in a gel drier before exposure to an X-Ray film.

2.28 Analysis of Sequencing Gels.

The sequencing gels were read manually and the data compared with the published sequence of the entire *HGPRT* gene (Edwards et al., 1990) or to a control sample of the amplified exon of interest for the *DMD* gene.

2.29. Fluorescent Automated Sequencing of Double-Stranded PCR Products Using Taq Terminator Chemistry and Cycle Sequencing.

2.29. 1 Preparation of DNA for Cycle Sequencing.

Double-stranded PCR products were prepared as described in2.5,2.6 and 2.7. 5 µl were run on a 1% agarose mini-gel to check for the presence of a product of the correct size. If one clean band was present, the remaining product was run through a Pharmacia Microspin S-400 column (Pharmacia Biotech). If more than one band was present , the desired band was isolated by cutting from a 1% agarose mini-gel as described in 2.14.2.

2.29.2 Cycle Sequencing of PCR Products Using Taq Terminator Sequencing.

7µl of the cleaned PCR product was mixed with 3.2pm of one of the primers used for the initial PCR. The volume was made up to 10.5µl by addition of 9.5 µl of PRISM Ready Reaction Dyedeoxy Terminator Cycle Sequencing premix added (Applied Biosystems). Cycle sequencing was carried out using a PTC-100 Programmable Thermal Controller (MJ Research Inc.) involving 25 cycles of denaturation at 96^{0}C for 15 secs, annealing at 50^{0}C for 15 secs and extension at 60^{0}C for 4 mins.

2.29.3 Cleaning of Sequencing Reactions.

80µl of dd$H_2$0 was added to the sequencing reactions and two phenol/chloroform/water (68:18:14) (ABI), extractions were carried out. The labelled DNA was then precipitated by the addition to the aqueous phase of 15µl of 2M sodium acetate (pH 4.5) and 300µl 100% ethanol followed by standing at -70 ^{0}C for 15 mins. The pellet was then collected by spinning for 15 mins at full speed in a microcentrifuge and washing in 70% ethanol

before the pellet was dried for 5 mins. in a vacuum dryer. 4µl of loading buffer(appendix 1) was added before the sample was denatured at 92^{0}C for 2 mins, quenching on ice and loaded on a 5.75% denaturing sequencing gel on the ABI automated sequencing apparatus.

2.29.4 Running of Sequencing Reactions on an ABI automated Sequencer.

5.75% denaturing gels were prepared and run by Robert McFarlane according to the parameters suggested by ABI. The gel matrix was degassed before pouring onto plates which had been stringently cleaned to avoid smearing and background mess which could be misinterpreted by the scanner as fluorescent signal. The gels were prerun before loading and scanned for signal artefacts caused by dirt on the plates.

2.29.5 Analysis of Sequencing Gels.

The model 373A DNA sequencer system consists of two separate instuments, the 373A sequencer and a Mackintosh Centris 650 computer. The sequencer comprises of the microprocessor-controlled electrophoresis and fluorescent detection system and the computer receives data from the sequencer and processes it. Data was presented in several forms but was collected in the form of analysed data which was presented as a chromatogram with four coloured peaks representing the four bases with the base designation also given above each peak. Heteroduplexes represented by double peaks were identified by eye and confirmed by sequencing in the opposite direction.

CHAPTER 3

RESULTS

3.1 Lesch-Nyhan Syndrome.

3.1.1 Optimisation of the PCR reactions.

The initial stage of the mutation detection methods used in this study involved amplification of particular segments of the *HGPRT* gene. The amplification reactions required optimisation, involving modification of one of two parameters, the reaction components and the cycling parameters. As the oligonucleotide primers used were those suggested by Gibbs et al., (1990), the initial conditions used were as suggested and these are described in section 2.5. The PCR initially used the primer set which amplified exon 1 and these conditions did not produce an amplification product. Various parameters were then modified as follows.

a) Alteration of the Reaction Components.

The reaction components in the PCR reaction were altered individually while other components remained constant. Primarily the reaction buffer, dNTP stock solutions and Taq polymerase were checked by using several different stock solutions of each. No amplification was seen using any combinations of solutions.

The DNA quality and concentration were then analysed. Increasing concentrations of DNA from 50 ng to 1 µg were used. No amplification at any concentration was seen . The quality of DNA was then under suspicion and aliquots of the control samples being used for amplification were run on a 1% agarose gel. The DNA did not appear to be degraded. To rule out the possibility of contaminants in the DNA which would inhibit amplification such as phenol from the DNA extractions, aliquots of DNA from colleagues which were known to be good templates were used in the reaction and still no amplification was seen.

Gibbs et al. (1990), suggested the addition of 6.7mM magnesium chloride to the reaction mix. The reactions were then carried out in the absence and with increasing concentrations of magnesium chloride to a final value of 5x the suggested value. No amplification was seen at any

manufacturers

concentration. It was also suggested that the cosolvent dimethyl sulfoxide be included in the PCR reaction. Cosolvents have been shown to overcome some of the difficulties seen in a PCR reaction due to the hindering effects of secondary structures which arise due to G/C rich DNA. Varying concentrations of DMSO were added to the reaction mix from 1% to 20% and still no amplification was seen. Similarly TMAC was added up to a value of 10% of the reaction mix and again no difference was noted.

Primer concentration has been shown to be very important to the efficiency of the PCR reaction. The primer concentrations were altered an values between 10p**M** and 1**m**M were used with no concentration variation producing an amplification product.

The final reaction component to be looked at was the concentration of dNTPs used in the reaction mix. The concentration suggested by Gibbs et al., (1990), was very high compared to other PCR reactions which were working successfully in the laboratory. Therefore the concentration of dNTPS were altered and a range of concentrations were used with no amplification product being seen.

b) Cycling Parameters.

The modifications in the reaction components were carried out while the cycling parameters were held at the suggested temperatures and times with an annealing temperature of 57^0C. The second set of modifications involved varying the annealing temperatures from 52^0C to 65^0C. Amplification did not occur at any of the annealing temperatures. Different reaction mixes were then tried at the range of temperatures and cycle numbers and still no amplification was seen.

The initial publication used a Perkin-Elmer Cetus thermocycler for the reactions and the previous described manipulations were carried out on a Techne 1. The reactions were then tried on a Perkin-Elmer/Cetus and a Techne PH3 but no amplification occured.

c) Oligonucleotide Primers.

The lack of amplification appeared to be due to the deficiency of a major component of the PCR reaction mix. One of the most important components are the oligonucleotide primers and two further possibilities existed for a deficiency in this component. Firstly, the primer sequence could be wrong in either a human error in ordering of the primers or in a mistake in their manufacture. Secondly, the primers may have been impure, degraded or contained many incomplete sequences making them unsuitable for amplification. To exclude these possibilities the primers were firstly run on a 5% denaturing polyacrylamide gel to identify either a discrete band if the primers were of a pure quality and not degraded or a smear if they were of poor quality. A discrete band was seen and is shown in figure 3.1 .

If the primer sequence was wrong or not compatible for amplification any range of amplification conditions could be tried and no amplification would ever occur. Therefore a second primer sequence was manufactured and purified by HPLC and these primer sequences flanked exon 2. Different reaction conditions and cycling parameters were used with the new primer set and on decreasing of the dNTP concentration and using an annealing temperature of 57^0C an amplification product of the correct size was seen on one occasion. However, this amplification was not reproducible although a degree of smearing was seen on the gel after electrophoresis of several reaction mixes and conditions (figure 3.2) . This suggested that some non-specific amplification had occured using the exon two primer set and that another critical reaction component other than the primers used was inhibiting amplification.

d) PCR Buffer.

Gibbs et al. (1990), suggested an unusual reaction buffer for use in the PCR reaction which was made up in the laboratory and was not similar to the composition of the buffer supplied with the Taq polymerase. Therefore either the buffer had not been made correctly which was unlikely as several different batches were prepared and used ,or the buffer was not suitable for amplification under the conditions used. Parallel reactions were set up using both exon one and two primer sets at different annealing temperatures with low and high dNTP concentrations and with the suggested laboratory

Figure 3.1: Electrophoresis of *HGPRT* oligonucleotide primers. Both lanes labelled 1 represent the sense and antisense exon 1 primers and both lanes labelled 2 represent the sense and antisense exon 2 primers.

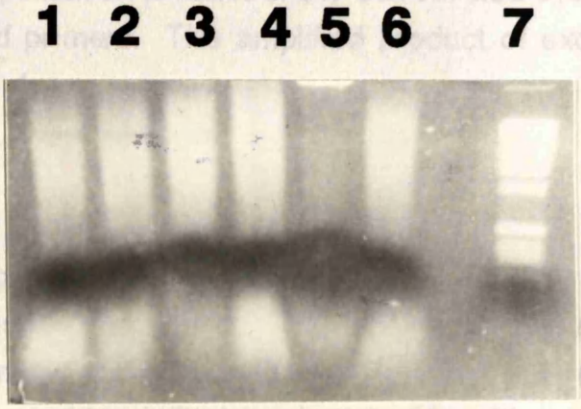


Figure 3.2 : Products of a pre-optimised PCR reaction run on an agarose gel. Lanes 1-6 represent amplification of exon 2 of the HGPRT gene, using different PCR parameters and conditions. Lane 7 contains the 1kb ladder.

made buffer and with the reaction buffer supplied by Boeringer. At an annealing temperature of 56^{0}C a band of the correct size as well as non-specific products were seen with exon 2 primers, with the low concentration dNTP mix and the Boeringer supplied buffer . The exon 1 amplification reaction however showed many non-specific bands of the wrong size (figure 3.3) . Different annealing temperatures were used for amplification of exon two and for primers made for exon five and amplification occurred optimally at an annealing temperature of 61^{0}C for all exons except exon 1 which would not produce an amplification product of the correct size even with the use of new HPLC purified primers. The amplified product of exons two to nine are shown in figure 3.4.

3.1.2 PCR Failure.

Despite optimisation, at several stages during the study individual exons and all the exons simultaneously failed to amplify on several occasions. If one exon failed to amplify while the others showed no such problem the amplification could usually be reinstated by either cleaning of the primers by ethanol/salt precipitation or if that failed, manufacture of a new primer set which was either HPLC purified (Oswell DNA) or purified after manufacture in the department by purification through an OPC column (ABI). Primers which were manufactured in the department which were not purified in such a way were generally unreliable in this PCR reaction and produced either non-amplification or became useless after a few freezing and thawing events. Therefore the purity and quality of the primers were obviously very important to this non-robust PCR.

If all exons failed to amplify simultaneously then all the reaction components were checked until the problem area was identified. This involved primarily changing the dNTP stock solutions or PCR buffer or Taq polymerase. If none of these components were faulty a control template DNA which was working for other PCR,s in the lab was used and if the DNA template was the faulty component, it was cleaned up by either phenol extraction or ethanol precipitation. A further problem area on two occasions was a contaminated double-distilled water source. The supply contained some form of contaminant which was found to inhibit both restriction enzyme cutting capacity and Taq polymerase in the less robust PCR reactions. This was found by the reinstatement of amplification following use of bought

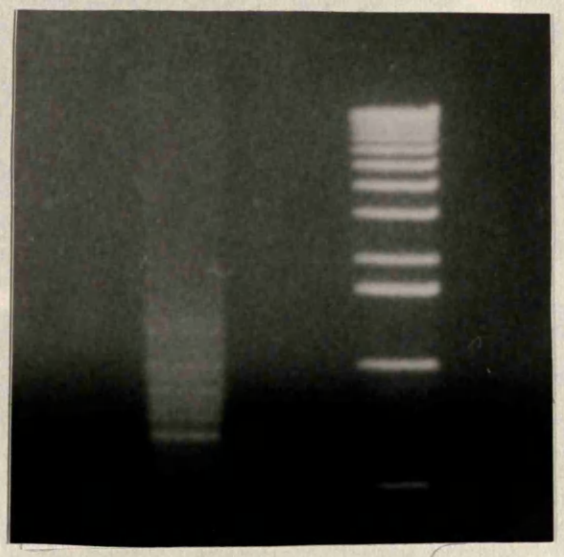


Figure 3.3: Agarose gel showing the amplification products from a PCR using exon 1 primers after optimisation of the conditions. Lane 1 shows the non-specific products obtained from the exon 1 primers and lane L shows a 1kb ladder.

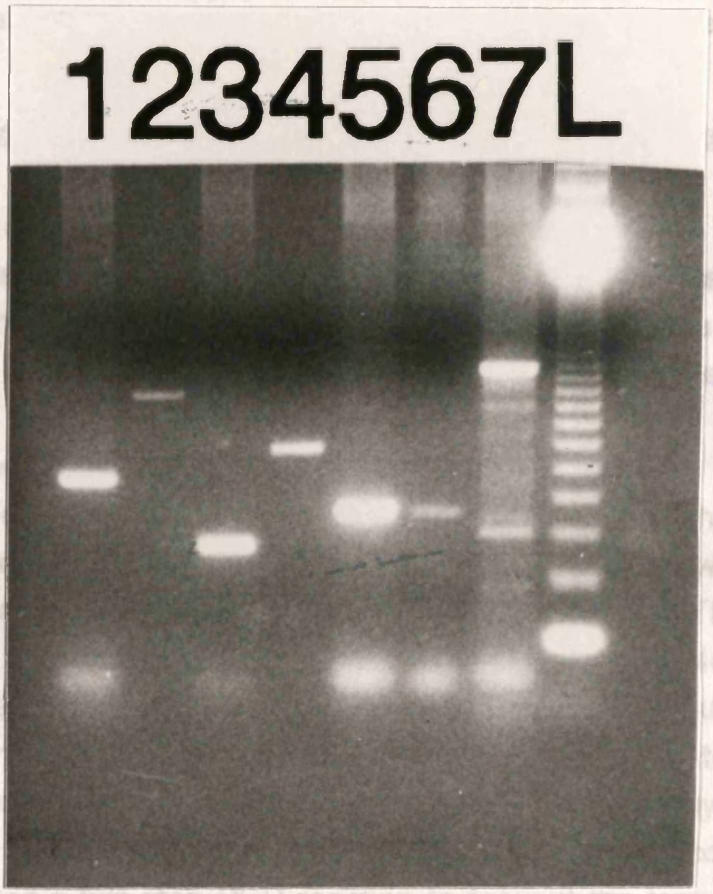


Figure 3.4 : Agarose gel showing amplification products from PCR reactions utilising primer sets from exons 2-9. Exons 7 and 8 are amplified as a single amplification product. Lanes 1-7 contain exons 2-9 with exons 7/8 as one product shown in lane 6. A 123bp ladder is shown in lane L.

bottled ddH_2O. The final cause for lack of amplification was contamination of the reaction tubes or the tips used to set up the reaction. On two occasions these were found to have been contaminated by rust in the autoclave. As a positive control for the viability of the reaction components, part of the CF gene was amplified. This is a very robust PCR, which if failed, indicated a fault with a component which if then altered allowed reinstatment of amplification.

3.1.3 Optimisation of Heteroduplex Detection on Hydrolink Gels.

A protocol for heteroduplex detection using hydrolink gels (HDHG) was optimised using a panel of 10 known mutations present in seven different exons. The base changes in these mutated exons and the size of the amplified fragment which contains them are shown in Table 3.1.

The mutation HGPRT Farnham (C486G) amplification fragment was primarily used to establish the optimum conditions. Due to the presence of one X chromosome in males a potential heterozygous state was created by addition of normal male DNA to the PCR reaction. The conditions suggested by Keen et al., (1991), which included an extra denaturing/renaturing step after PCR of 94^0C for 2.5 minutes 0^0C for 2 minutes then room temperature for 60 minutes, were initially used for enhancing the formation of heteroduplexes. This methodology did not show the presence of heteroduplex bands. Keen et al. (personnel communication) then suggested a two step PCR reaction to prevent the formation of ghost bands and different methods of heteroduplex formation after PCR were assayed. The optimum results were obtained using a two step PCR with a final denaturation/renaturation step of 94^0C for 30 seconds and 70^0C for 1-16 hours. Figure 3.5.1 shows the ethidium bromide stained hydrolink gel showing the amplified exon of the *HGPRT* gene containing this mutation C486G (HGPRT Farnham) after heteroduplex formation.

The remaining nine mutations were then analysed in a similar way. Different parameters were altered to find the optimum conditions for detection of each mutation. The optimum thickness of gel used was found to be 1.5mm thick, and the mutations were run either on a 20cmx20cm or 20cmx30cm gel depending on the size of the amplified fragment that contained the mutation. Different electrophoresis conditions were also applied depending on the size of the fragment and these conditions are summarised in Table 3.1

Table 3.1 . Summary of known mutations detected by heteroduplex detection on hydrolink gels, and the optimum gel conditions used for the detection of each mutation. The nucleotide changes present are indicated. a-indicates an insertion, b-indicates a deletion, + indicates detection of a heteroduplex and - indicates no detection of a heteroduplex.

MUTATION	SEQUENCE CHANGE	FRAGMENT SIZE(bp)	GEL CONDITIONS	DETECTED
HGRRT CODICOTE	T298[a]	1059	1.5mm thick, 20x30cm, 250V-7h	+
HGPRT CHELTENHAM	GT[b] 288-289	1059	1.5mm thick, 20x30cm, 250V-7h	+
HGPRT MASHAD	A59T	572	1.5mm thick, 20x20cm, 250V-6h	+
HGPRT SHEFFORD	C151T	1059	1.5mm thick, 20x30cm, 250V-7h	+
HPRT BANBURY	G152C	1059	1.5mm thick, 20x30cm, 250V-7h	+
HGPRT RUNCORN	T395C	707	1.5mm thick, 20x20cm, 250V-7h	+
HGPRT FARNHAM	C486G	441	1.5mm thick, 20x20cm, 250V-6h	+
HGPRT NORTH MYMMS	C508T	433	1.5mm thick, 20x20cm, 250V-6h	+
HGPRT MARLOW	C527T	433	1.5mm thick, 20x20cm, 250V-6h	+
HGPRT MARLOW	C527T	1533	1.5mm thick, 20x30cm, 250V-8h	-
HGPRT READING	G617A	1278	1.5mm thick, 20x30cm, 250V-8h	+

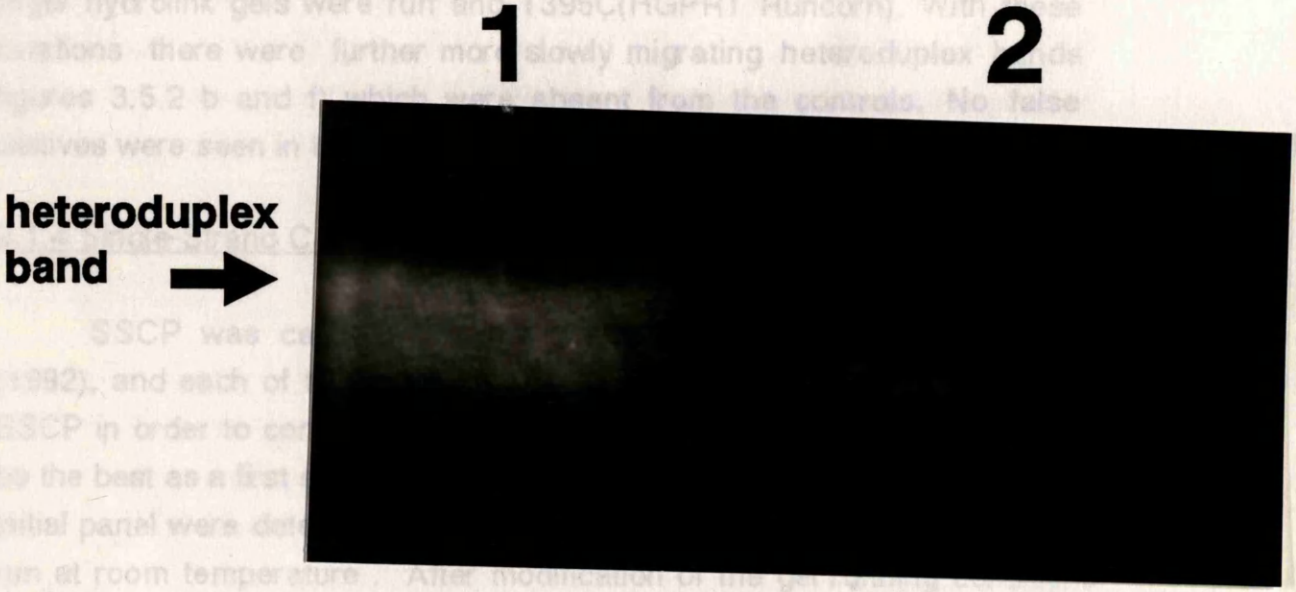


Figure 3.5.1 : Detection of a heteroduplex on hydrolink gels in exon 6 of the Index case with the mutation HGPRT Farnham. Lane 1 shows the doublet heteroduplex and homoduplex band apparent when control DNA was added to the reaction tube with the mutant DNA. Lane 2 shows the single band product of a PCR reaction where control DNA was mixed with DNA from a normal individual .

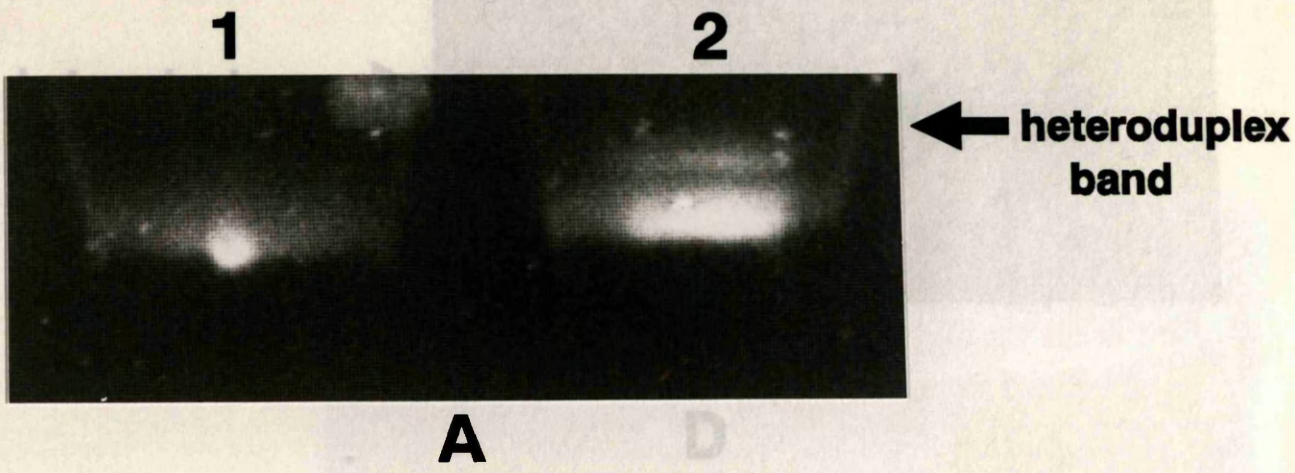
Heteroduplex detection using hydrolink gel electrophoresis initially detected nine of the ten mutations represented in the panel. The patterns obtained are shown in figure 3.5.2a-h. Some of the heteroduplexes were less convincing(c and d), and unequal loading of the sample and control caused less convincing results(a,e and f). HGPRT Marlow (C527T) present in an amplification fragment of 1553 bp in size was not detected at first but by using primers which produced an amplification product of 433bp in size which contained the mutation, it could be detected (Figure 3.5.3). With the majority of mutations a second slightly slower migrating band present close above the first was apparent representing the formation of a heteroduplex between the mutant and control DNA, for example with C486G (HGPRT Farnham)(Figure3.5.1).This is the pattern seen with the majority of mutations with the exception of GT288-289(HGPRT Cheltenham) when longer hydrolink gels were run and T395C(HGPRT Runcorn). With these mutations there were further more slowly migrating heteroduplex bands (figures 3.5.2 b and f) which were absent from the controls. No false positives were seen in this gene with the panel of known mutations.

3.1.4 Single-Strand Conformational Polymorphism Analysis (SSCP)

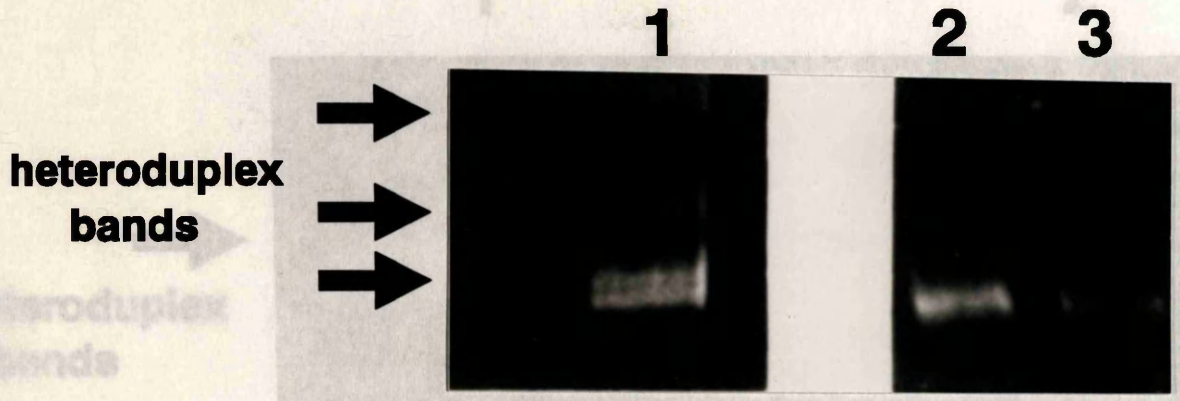
SSCP was carried out using the protocol described by Kataki, (1992), and each of the mutations identified by HDHG were analysed by SSCP in order to compare the two techniques and to identify which would be the best as a first screening strategy. None of the ten mutations from the initial panel were detected on a non-denaturing gel containing 10% glycerol run at room temperature . After modification of the gel running conditions two mutations, HGPRT Mashad present in the 572 bp exon 2 amplification fragment and HGPRT Marlow present in the 433 bp exon 7 amplification fragment were detected when the glycerol concentration was reduced to 5% and the gel run at 4^0C (Figure 3.6 and 3.7). However these mutations were not consistently detected.

The remaining mutations could not be detected by this protocol so three exons (3, 5 and 7) were digested with relevant restriction enzymes , labelled and subjected to SSCP analysis. The results obtained for each exon were confusing and extra bands were apparent even in control lanes making interpretation of the results difficult. The detection rates of the two protocols, HDHG and SSCP are summarised in figure 3.8.

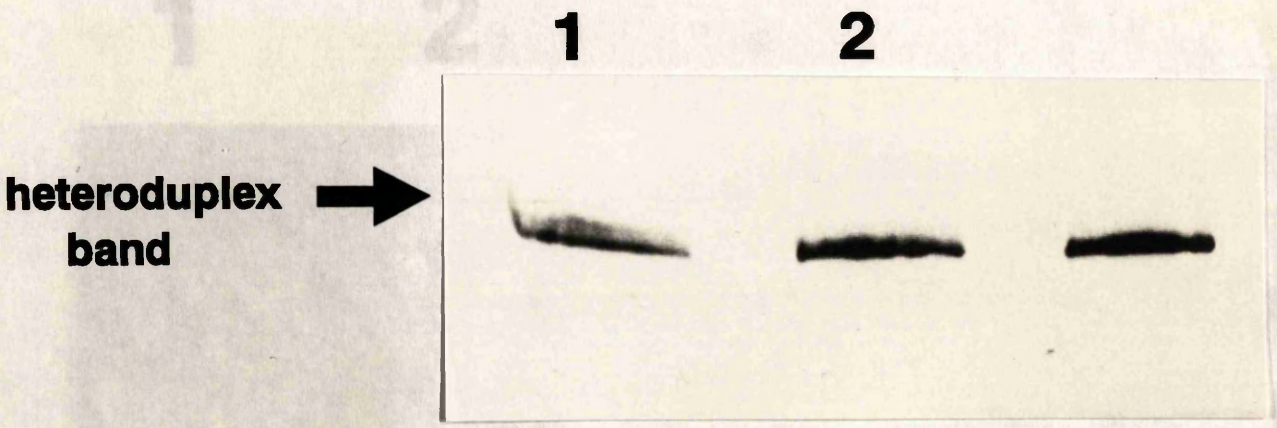
Figure 3.5.2 : Hydrolink gels showing heteroduplex detection of previously described mutations. The following mutations were detected and are shown in figures a-h respectively ;(a) exon 2 of the index case with the mutation HGPRT Mashad, the index case DNA is in lane 2 and normal DNA in lane 1 ; (b) exon 3 of the index case with the mutation HGPRT Cheltenham, the index case DNA is shown in lane 1, with normal DNA in lanes 2 and 3; (c) exon 3 of the index case with the mutation HGPRT Codicote, the index case DNA is shown in lane 1 and normal DNA in lane 2;(d) exon 3 of the index case with the mutation HGPRT Banbury and the index case DNA is shown in lane 1, with normal DNA in lane 2; (e) exon 3 of the index case with the mutation HGPRT Shefford, the index case DNA is shown in lane 1, with normal DNA in lane 2; (f) exon 5 of the index case with the mutation HGPRT Runcorn,the index case DNA is shown in lane 2, with normal DNA in lane 1; (g) exon 7 of the index case with the mutation HGPRT North Mymms,the index case DNA is shown in lane 1, with normal DNA in lane 2;(h) exon 9 of the index case with the mutation HGPRT Reading, the index case DNA is shown in lane 1, with normal DNA in lane 2. The DNA from both the index case and the normal DNA is mixed with a control DNA sample. The heteroduplex bands are indicated by an arrow and the actual base changes are shown in table 2.4.


1
2
heteroduplex band

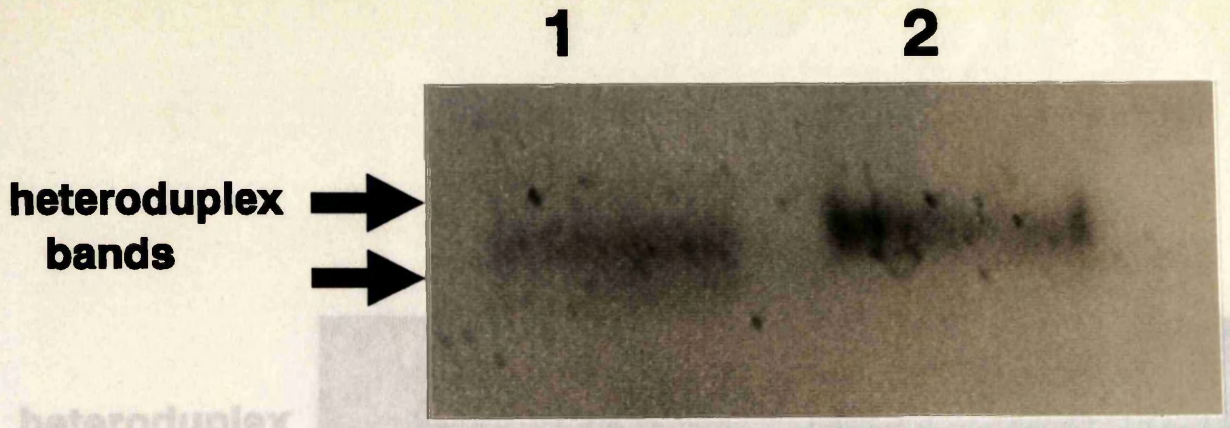
A


1
2
3
heteroduplex bands

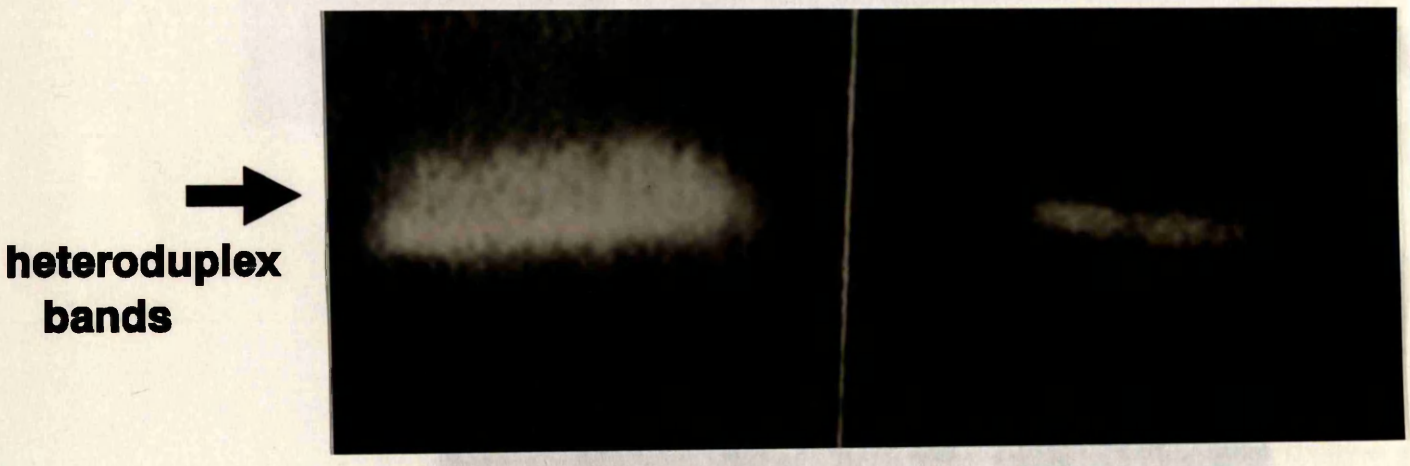
B


1
2
heteroduplex band

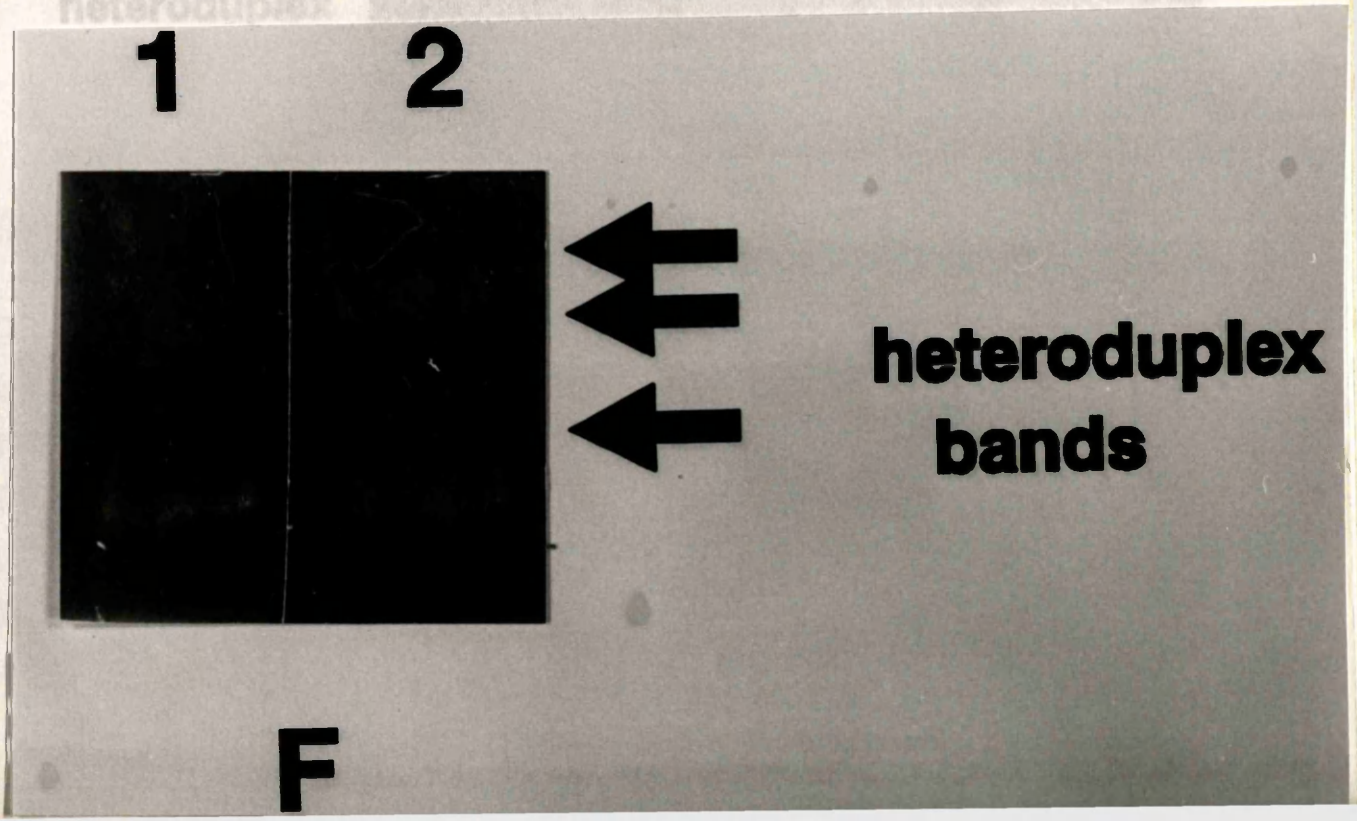
C


1
2
heteroduplex
bands

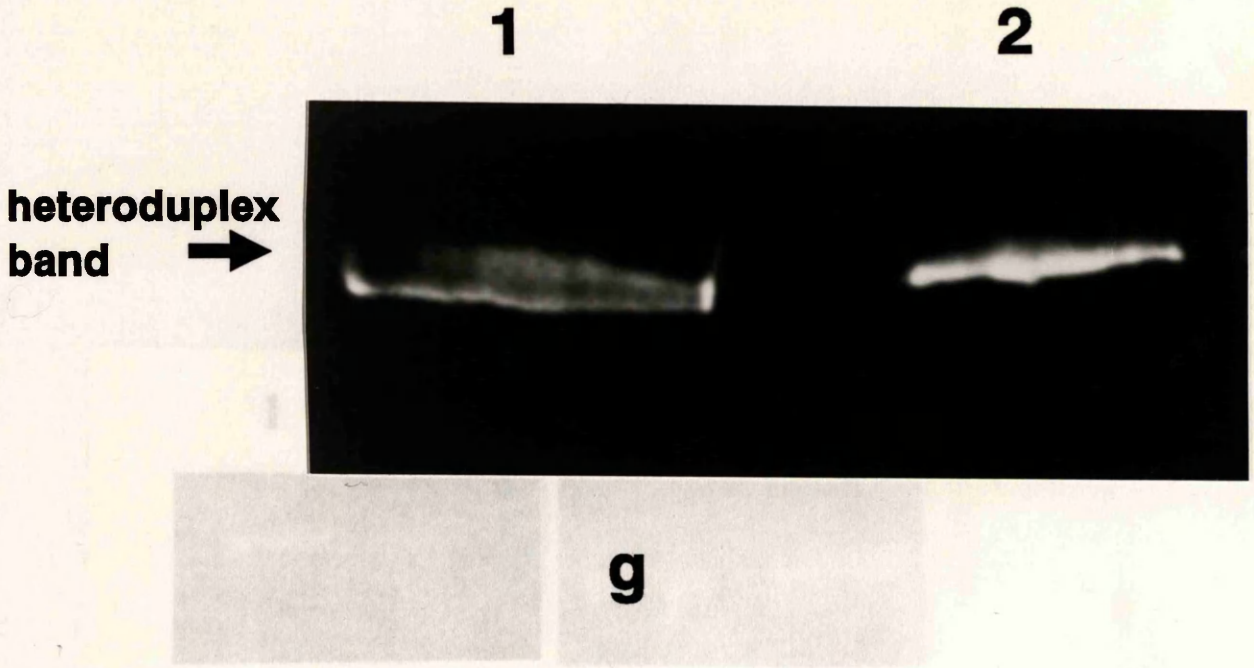
D


1
2
heteroduplex
bands

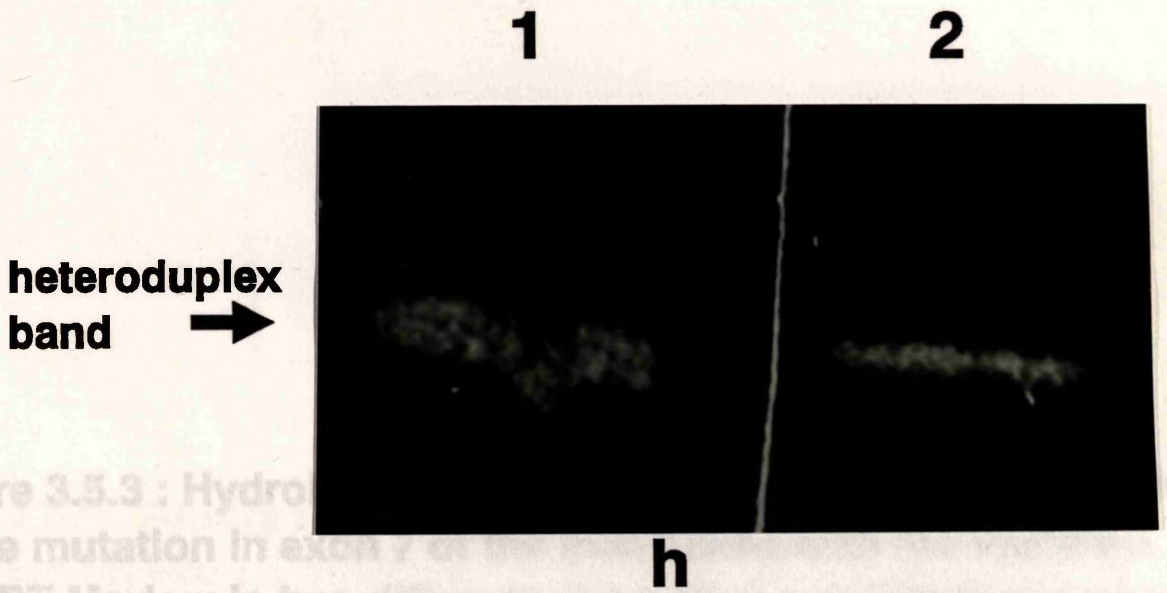
E


1
2
heteroduplex
bands

F



g



h

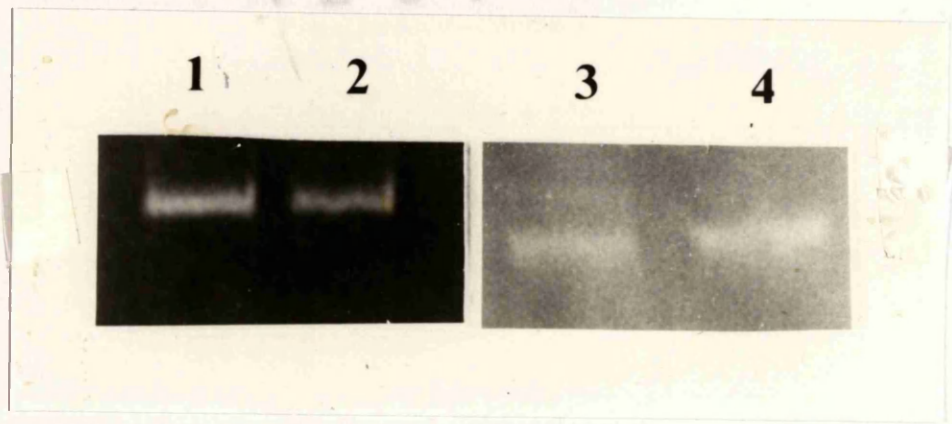


Figure 3.5.3 : Hydrolink gels showing non-detection and detection of the mutation in exon 7 of the index case with the mutation HGPRT Marlow in two different sized PCR amplification fragments. Lanes 1 and 2 show the affected and control samples respectively, after amplification with normal DNA using primers which produce a PCR product of approximately 1.5 kb. Lanes 3 and 4 show the affected and control DNA samples respectively, after amplification with normal DNA, using primers which produce an amplification product of 433bp.

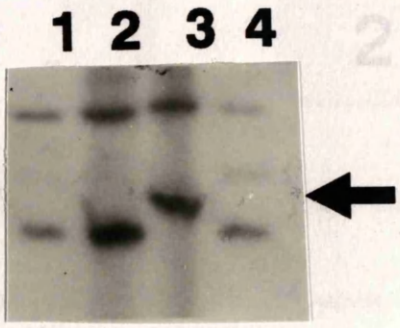


Figure 3.6 : Autoradiograph showing the results of SSCP analysis on exon 2 from the index case with the mutation HGPRT Mashad (lane 3) aginst exon 2 from normal individuals (lanes 1,2 and 4). The band shift is indicted by an arrow.

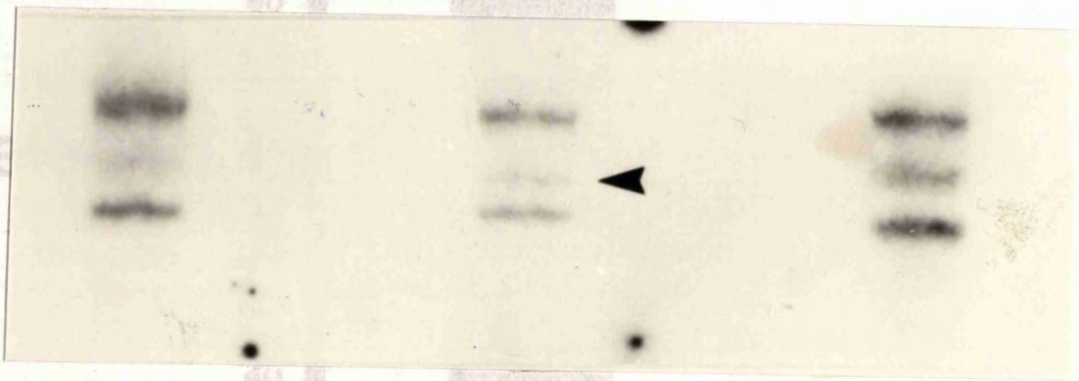


Figure 3.7 : Autoradiograph showing the results of SSCP analysis on exon 7 of an index case with Lesch-Nyhan syndrome. This shows exon 7 of the index case with the mutation HGPRT Marlow. The index case is shown in lane 2 with a band shift indicated by an arrow , relative to two normal males shown in lanes 1 and 3.

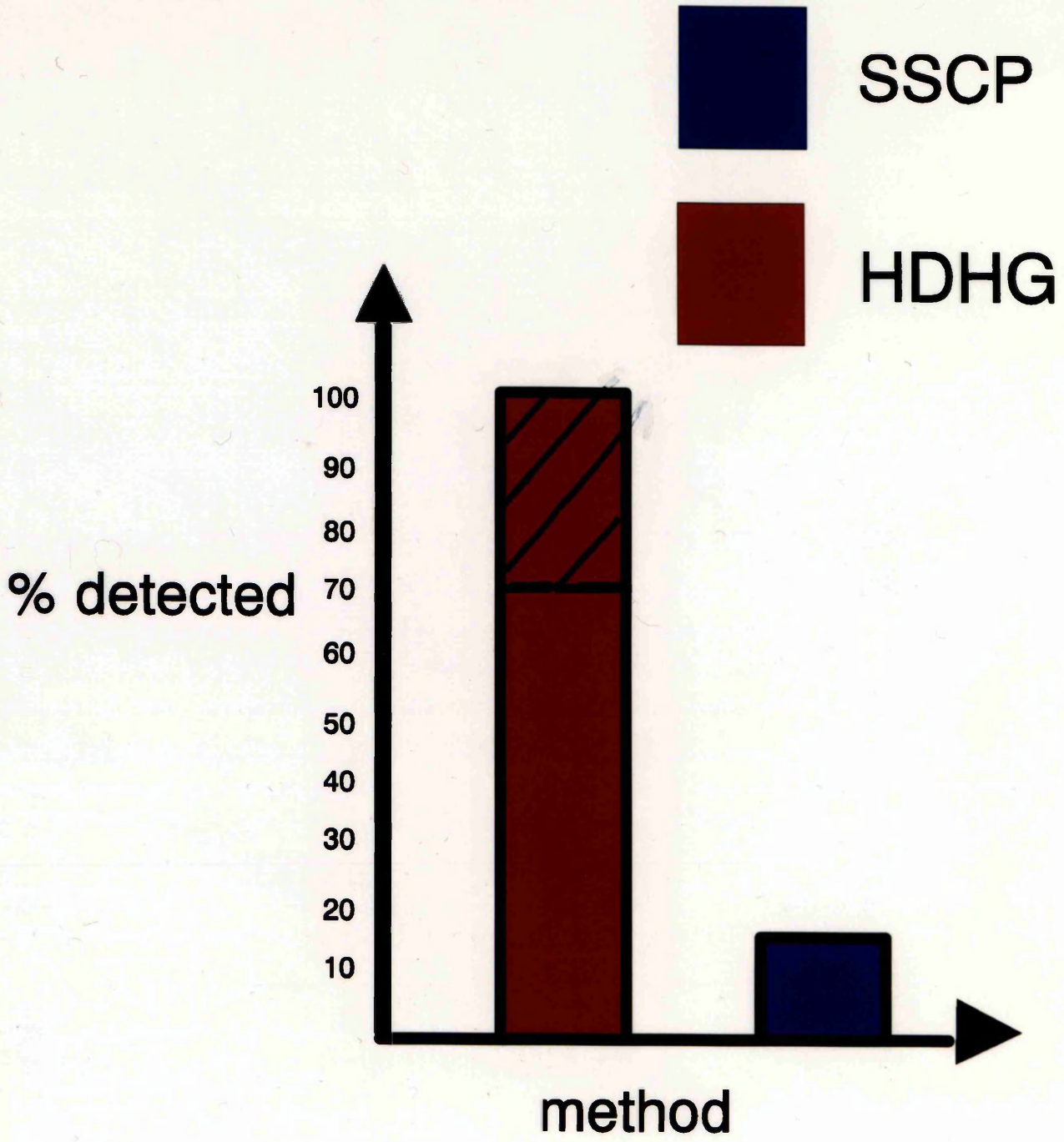


Figure 3.8 : Graph showing the relative efficiency of the two mutation screening strategies(SSCP and HDHG) in detecting ten known mutations in the HGPRT gene.

The solid red box indicates the base changes which were definitively detected by the HDHG protocol and the shadedred box indicated base changes which were detected butwhere the heteroduplexes were less clear and may be missed if the presence of the mutation was not known.

3.1.5 Optimisation of Chemical Cleavage of Mismatches.

This protocol was optimised using two mutations, The cystic fibrosis G551D mutation which results from a G to A substitution and a mutation in intron 44 of the dystrophin gene (Kataki,1992) which also resulted in a G to A substitution. These changes resulted in an A:C mismatch which should be detected by piperidine cleavage after modification of the mismatch using hydroxylamine.

The original protocol based on that suggested by Cotton et al., (1988), is described in 2.19.1, where the probe was prepared by labelling of a PCR product and was applied to the G551D mutation after PCR of the mutation containing region . However, neither homoduplex or cleaved heteroduplex band were seen in the control or test DNA lanes. Different quantities of labelled probe of 1x - 6x the suggested levels were then mixed with corresponding quantities of unlabelled test DNA and the protocol repeated. On this occasion uncleaved bands were seen in the control and test lanes with the strength of the signal varying with the quantity of probe added (figure 3.9. lanes 1). However, no cleavage product was seen and increased quantities of DNA present were presumed to be interfering with the reaction inhibiting heteroduplex formation, modification and/or cleavage.

Alternative methods for probe preparation involving labelling of the primers before PCR and incorporation of radioactivity during PCR were then used in order to obtain a more efficiently labelled probe. These are outlined in 2.19.2. CCM was carried out using these probes and while uncleaved bands were visible on the autoradiograph at lower concentrations of probe still no cleavage was seen.

A modified CCM protocol was applied as is outlined in 2.19.2., using probes which had been made by incorporation of $^{32}P\alpha$-dCTP along the length of the probe during PCR. This altered protocol using siliconised tubes and different methods of probe preparation yielded two cleavage products after CCM and the autoradiograph is shown in figure 3.9., lanes 2. The protocol was repeated for the *DMD* mutation using a probe which had been made by PCR using γATP end-labelled primers and cleavage occured with only one cleaved band being visible as expected as the probe was end-

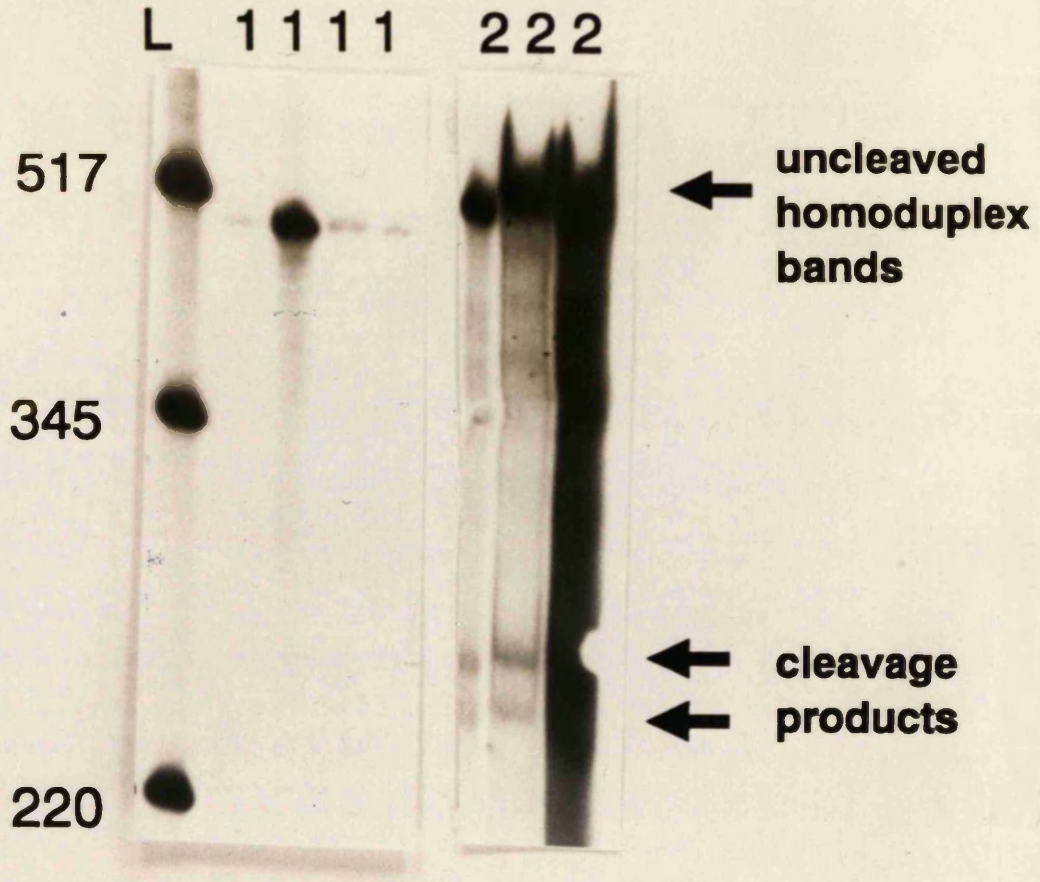


Figure 3.9 : Autoradiograph of CCM analysis of the cystic fibrosis G551D mutation using two different CCM methodologies. The lanes numbered 1 show CCM using method 1 described in chapter 2 and the lanes numbered 2 show CCM using method 2 as described in chapter 2. The probe was internally labelled , with the uncut homoduplex bands and the cleavage products being indicated by arrows. A labelled 1kb ladder is shown in lane L.

labelled at only one end (figure 3.10.). This method of CCM was then elected for use. Once again, unequal loading of the control lanes relative to the test sample in all the CCM results led to less clear results

3.1.6 Screening for Molecular Pathologies in Lesch-Nyhan Syndrome.

After optimisation of the various mutation detection protocols a strategy for detection of the molecular pathologies responsible for Lesch-Nyhan syndrome in 13 families where the mutation had not been characterised , was developed and is outlined in figure 3.11. After screening for large length mutations in individual exons of the *HGPRT* gene by PCR, the mutation containing exon was identified by heteroduplex detection using hydrolink gels before confirmation was obtained by CCM analysis and the exact nature of the mutation detetmined by sequencing.

3.1.7 PCR Amplification of Individual Exons of the *HGPRT* Gene.

The individual exons of the *HGPRT* gene were amplified as described in 2.5 and the products sized on an agarose gel against a 1kb ladder. The results of the PCR amplification from 13 families with Lesch-Nyhan syndrome where the molecular pathologies were unknown are shown in Table 3.2. The individual exons excluding exon 1 from 11 pedigrees were all analysed and from two pedigrees(10208 and 10213) there was insufficient DNA for amplification of exons 7/8 and 9. DNA from the index case was analysed where possible with the exception of pedigrees 10426 and 10208 where there was no available DNA from the index cases and the maternal DNA was used. For pedigree 10426 the mother was an obligate carrier.

Three abnormal PCR patterns were found. The DNA from the index case from pedigree 10427 failed to amplify for exons 7/8. An internal control was successfully simultaneously amplified and still no amplification product for exons 7/8 was seen. Different primer sets were designed and manufactured for amplification of exons 7 and 8 individually to discover whether either of the exons were deleted. Both primer sets produced an amplification product of the correct size. The original primer set was then used for amplification in conjunction with the new sets in an attempt to establish if a mutation was present at one of the primer sites. Amplification

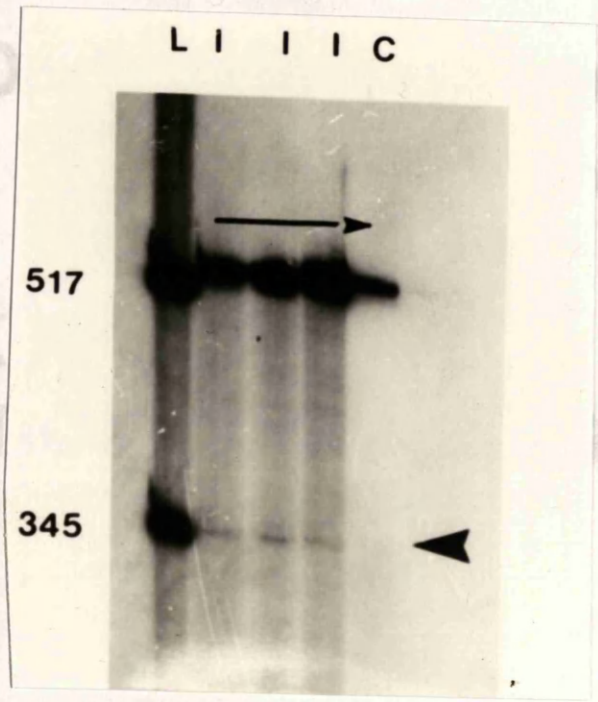


Figure 3.10 : Autoradiograph of CCM analysis of a polymorphic site in intron 44 of the dystrophin gene. Method two was used with increasing quantities of a 5'-end-labelled probe added to the test DNA (lanes 1). A normal DNA sample was mixed with the probe and analysed and the results shown in lane C. A labelled 1kb ladder is shown in lane L. The cleavage product is shown by an arrow.

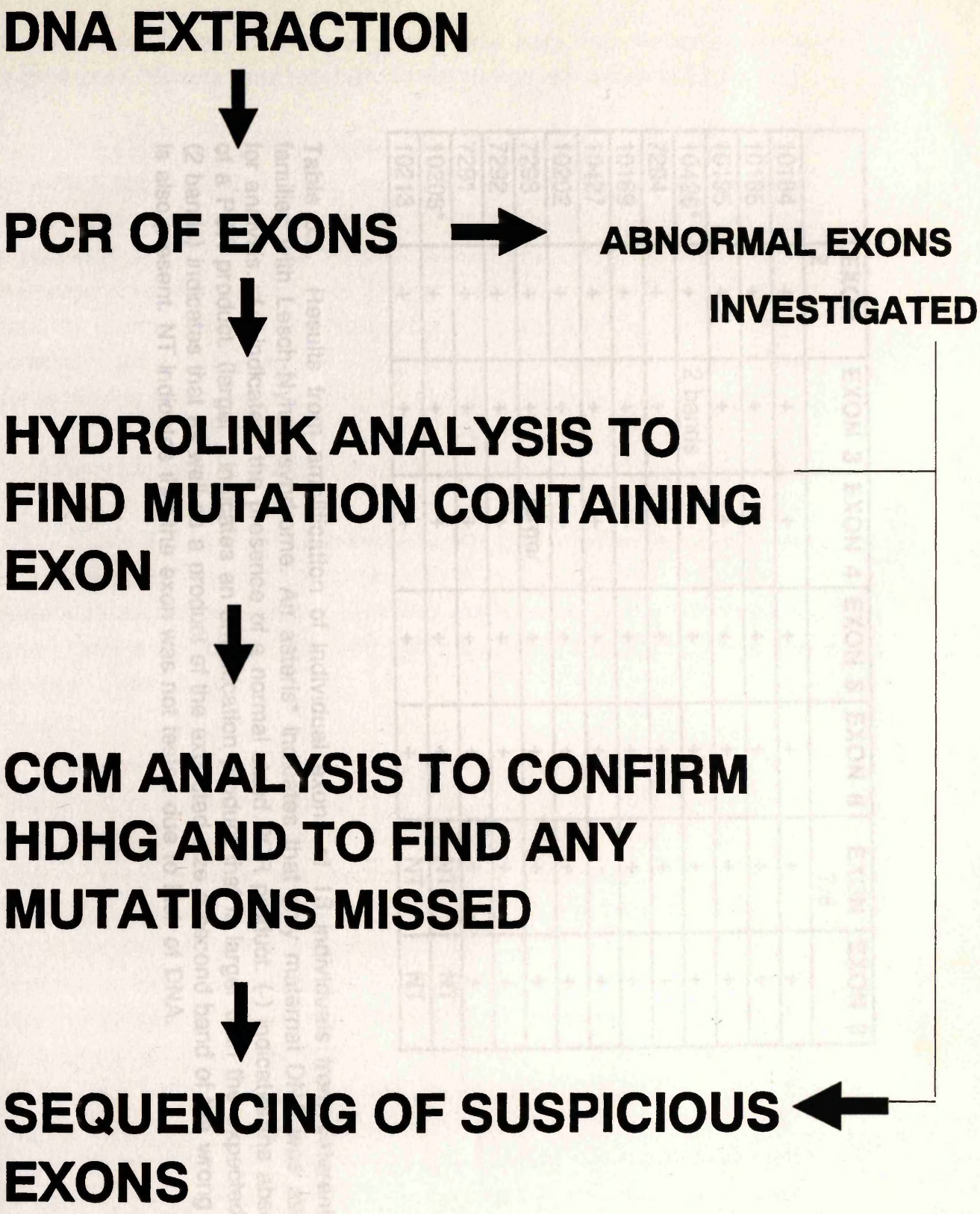


Figure 3.11: Schematic representation of the strategy for detection of molecular pathologies in the HGPRT gene.

	EXON 2	EXON 3	EXON 4	EXON 5	EXON 6	EXON 7/8	EXON 9
10194	+	+	+	+	+	+	+
10185	+	+	+	+	+	+	+
10195	+	+	+	+	+	+	+
10426*	+	2 bands	+	+	+	+	+
7294	+	+	+	+	+	+	+
10189	+	+	+	+	+	+	+
10427	+	+	+	+	+	-	+
10202	+	+	+	+	+	+	+
7293	+	+	larger	+	+	+	+
7292	+	+	+	+	+	+	+
7291	+	+	+	+	+	+	+
10208*	+	+	+	+	+	NT	NT
10213	+	+	+	+	+	NT	NT

Table 3.2. Results from amplification of individual exons of 13 individuals from different families with Lesch-Nyhan syndrome. An asteris* indicates that only maternal DNA was available for analysis. (+) indicates the presence of a normal sized PCR product. (-) indicates the absence of a PCR product. (larger) indicates an amplification product that is larger than the expected size. (2 bands) indicates that as well as a product of the expected size a second band of the wrong size is also present. NT indicates that the exon was not tested due to lack of DNA.

occured with all combinations of primer sets with the exception of the original exon 7/8 sets used together. These results are shown in figure 3.12.

The index case from pedigree 7293 showed an amplification product for exon 4 that was much larger than the expected product. This was sized on an agarose gel and was found to be approximately 370bp larger than expected (figure 3.13.). The obligate carrier from pedigree 10426 showed the presence of an extra band of a larger than expected size as well as the band of normal size, after amplification of exon 3. This band was not consistent but appeared in 3 of 6 amplifications. All other exons tested from the remaining pedigrees produced amplification products of a normal size.

3.1.8 Heteroduplex Detection in the *HGPRT* Gene Using Hydrolink Gels.

Exons 2-9 from the 13 individuals with Lesch-Nyhan syndrome were screened by HDHG for the presence of heteroduplexes indicative of nucleotide sequence changes. DNA was available for only a portion of the index case and where DNA was not available the maternal DNA was analysed (Table 3.3).

Heteroduplex bands were clearly present in four individuals, exon 2 of the index case from pedigree 7292, exon 6 of the index case from pedigree 7291, exon 3 from the pedigree 10213 and exon 5 from the mother of the index case from pedigree 10195 (figure 3.14. a, b, c and d respectively). Several other exons from other pedigrees showed bands that were suspicious but where a definite extra band was not clear. The other pedigrees showed no abnormal or heteroduplex bands but exons 7/8 and 9 were not analysed in some due to lack of DNA, and pedigree 7293 which had a larger exon 4 amplification product after PCR also showed no heteroduplex bands when the other exons were examined. For the pedigree 10427, no abnormal heteroduplex bands were seen. cDNA was available for the index case from this family and when run on an agarose gel was seen to be approximately 60bp larger than a normal cDNA. Once again, unequal loading of the control DNA relative to the test sample devalued the results particularly figures 3.14b and d.

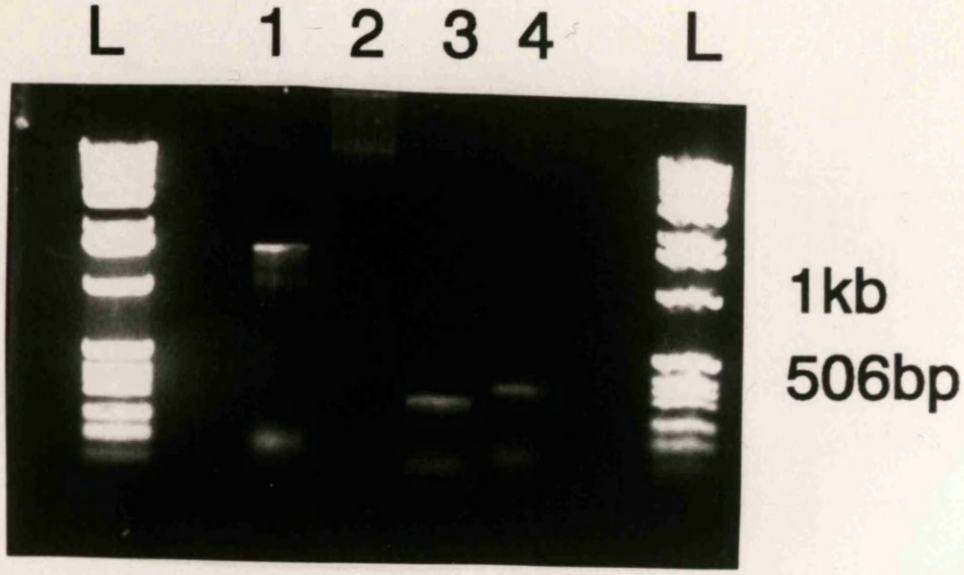


Figure 3.12: Ethidium stained agarose gel showing results of PCR amplification of exons 7 /8 from the index case from pedigree 10427 using different primer sets. Lanes L show a 1kb ladder. Lanes 1 and 2 show amplification using the exon 7/8 primer set described by Gibbs et al., 1990, amplifying control DNA and the index case DNA respectively. Lanes 3 and 4 show amplification of exons 7 and 8 independently, from the index DNA using primers designed by us.

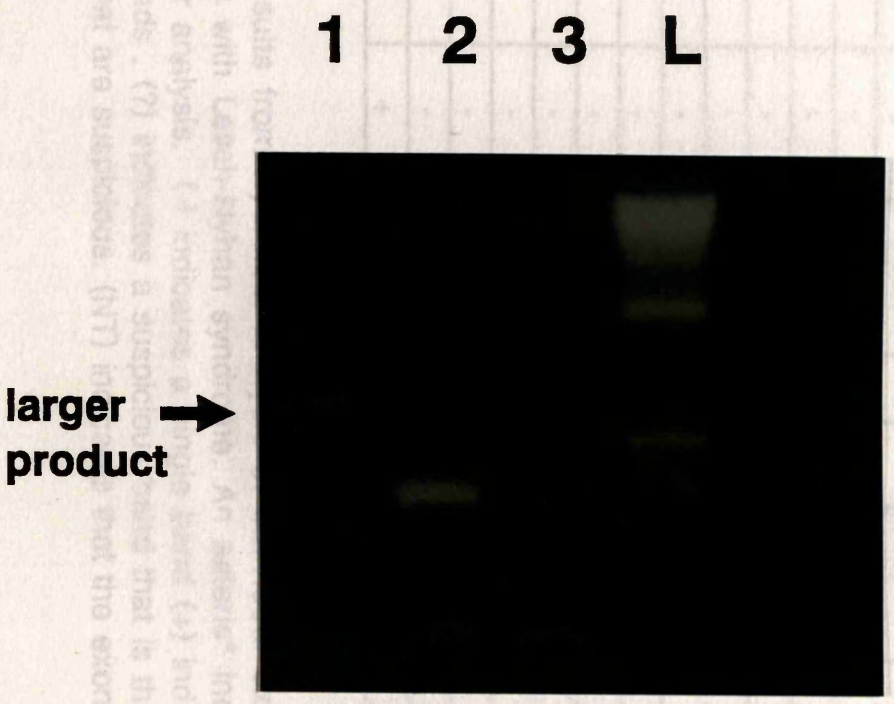
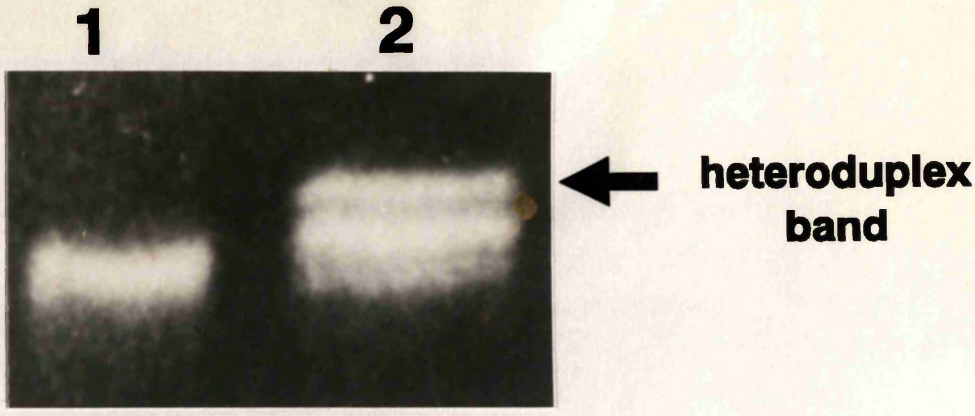


Figure 3.13 : Ethidium bromide stained agarose gel showing amplified exon 4 from a normal individual (2) and the index case (1) from the pedigree 7293. A 1kb ladder is shown in lane L.

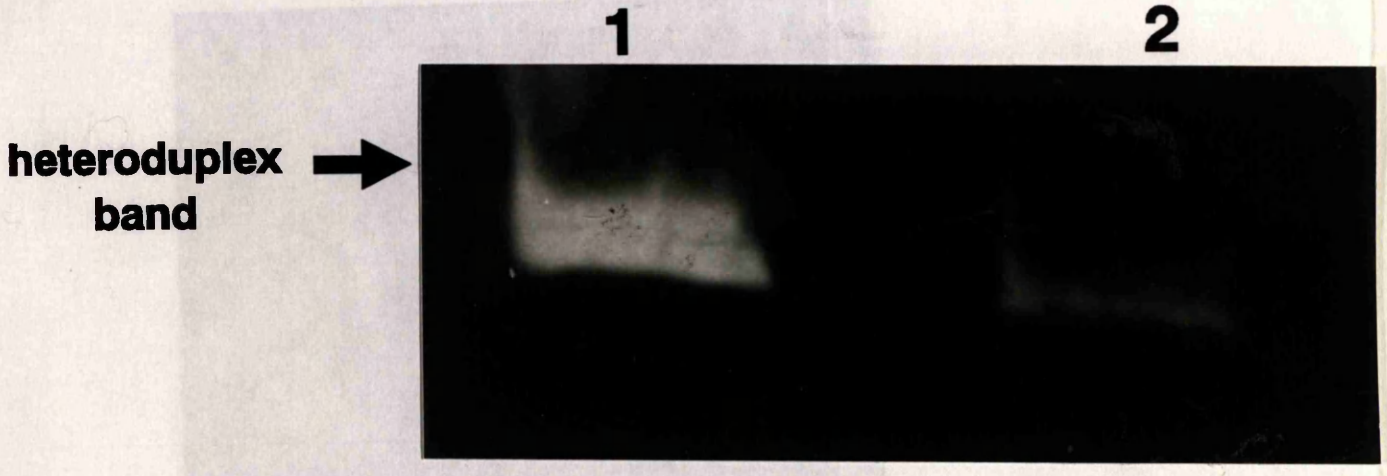
	EXON 2	EXON 3	EXON 4	EXON 5	EXON 6	EXON 7/8	EXON 9
10194*	?	?	-	?	-	-	-
10185*	?	-	-	-	-	-	?
10195*	-	-	-	+	-	-	-
10426*	-	-	-	-	?	-	?
7294*	-	-	?	-	-	?	?
10189	-	-	-	?		-	-
10427	-	-	-	-	-	-	-
10202	?	-	-	-	-	-	-
7293	-	-	NT	-	-	-	-
7292	+	-	-	-	-	-	-
7291	-	-	-	-	+	-	-
10208*	-	-	-	-	-	NT	NT
10213	-	+	-	-	-	NT	NT

Table 3.3 . Results from hydrolink analysis of individual exons from 13 individuals from different families with Lesch-Nyhan syndrome. An asteris* indicates that only rnaternal DNA was available for analysis. (-) indicates a single band (+) indicates the presence of heteroduplex bands . (?) indicates a suspicious band that is thicker than normal or the presence of extra bands that are suspicious. (NT) indicates that the exon was not tested.

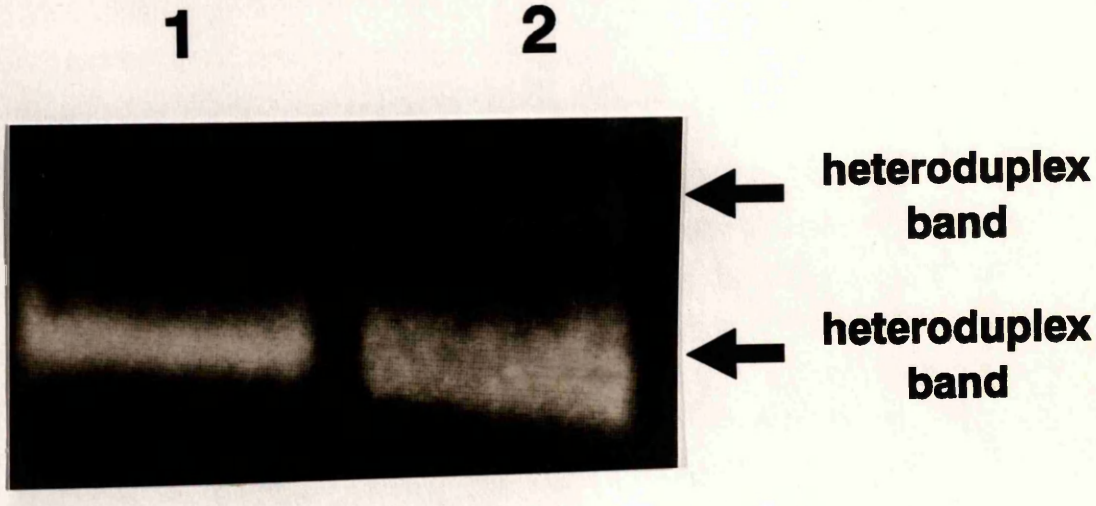
Figure 3.14 : Hydrolink gels showing the detection of previously unknown mutations responsible for Lesch-Nyhan syndrome in 4 families. Both the index case and control DNA samples were mixed with normal DNA. (a) exon 2 of the index case from the pedigree GLA 7292 (lane 2) and a normal exon 2 (lane 1); (b) exon 6 of the index case from the pedigree GLA 7291(lane 1) and a normal exon 6(lane 2); (c) exon 3 of the index case from the pedigree 10213(lane 2) and a normal exon 3 (lane 1); (d) exon 5 of the mother of the index case from the pedigree 10195 (lane 2) and a normal exon 5 (lane 1) . The Heteroduplex bands are indicated by an arrow.


1
2
heteroduplex
band

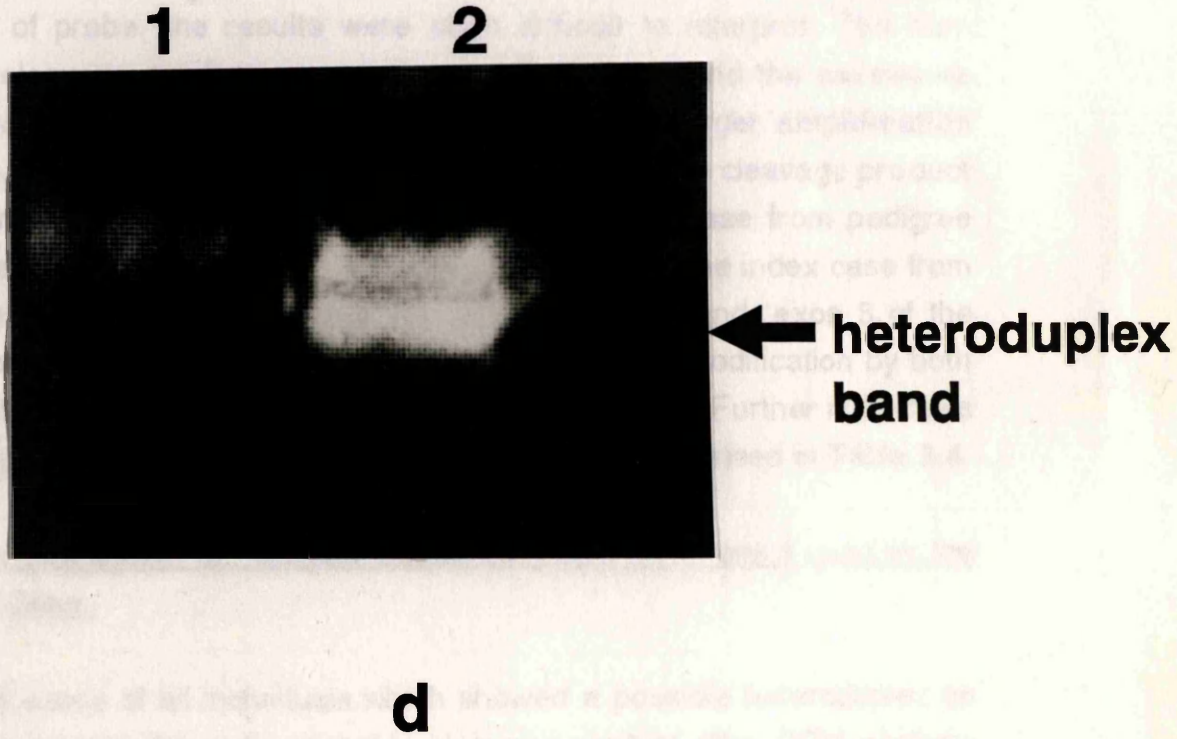
a


1
2
heteroduplex
band

b


1
2
heteroduplex
band
heteroduplex
band

c



d

3.1.9 Detection of Molecular Pathologies in the *HGPRT* Gene Using CCM Analysis.

Exons 2-9 were amplified from individuals from all the 13 pedigrees where DNA was available and CCM carried out after modification of potential mismatches with both hydroxylamine and osmium tetroxide. The results are summarised in Table 3.4.

Due to the nature of the CCM , degradation of probe, non-specific cleavage of regions of exons (particularly exon 5 and 7) and inadequate labelling of probe, the results were often difficult to interpret. The non-specific cleavage product constantly seen for exon 7 and the excessive background signals often obtained, particularly with larger amplification products, are shown in figures 3.15 and 3.16 . A definite cleavage product was seen in the following cases : exon 2 of the index case from pedigree 7292 after modification with hydroxylamine; exon 6 of the index case from pedigree 7291 after modification with hydroxylamine and exon 5 of the mother of the index case from the pedigree 10195 after modification by both osmium tetroxide and hydroxylamine; (Figures 3.17 a-c). Further individuals showed possible cleavage products and these are summarised in Table 3.4.

3.1.10 Optimisation of Manual Sequencing and Mutations Found in the *HGPRT* Gene.

All exons of all individuals which showed a possible heteroduplex on hydrolink gels or showed a probable cleavage product after CCM analysis were amplified by an asymmetric PCR to obtain a single-stranded product for direct sequencing. The sequences obtained were compared to the expected sequence published by Edwards et al., (1990).

Asymmetric products could not be obtained for all exons, with exons 2, 4, and 6 producing single-stranded products frequently while a single-stranded product appeared difficult to obtain for exons 3,5,7/8 and 9. A typical succesful asymmetric PCR showing the presence of single and double stranded DNA is shown in figure 3.18. Where a single-stranded product could not be obtained several modifications were applied to the asymmetric PCR in an attempt to obtain a product. Primarily the primer ratio of the limiting primer was altered and values of 1: 1/25, 1/50, 1/75, 1/100,

	EXON 2		EXON 3		EXON 4		EXON 5		EXON 6		EXON 7/8		EXON 9	
	H	O	H	O	H	O	H	O	H	O	H	O	H	O
10194*	-	-	-	-	-	-	?	?	-	-	-	-	-	-
10185*	-	?	-	-	?	-	-	-	-	-	-	-	-	-
10195*	-	-	-	-	-	-	+	+	-	-	-	-	-	-
10426*	-	-	-	-	-	-	-	-	-	-	-	-	-	?
7294*	-	-	-	-	?	-	-	-	-	-	-	-	-	-
10189	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10427	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10202	?	?	-	-	-	-	-	-	-	-	?	-	-	-
7293	-	-	-	-	NT		-	-	-	-	-	-	-	-
7292	+	-	-	-	-	-	-	-	-	-	-	-	-	-
7291	-	-	-	-	-	-	-	-	+	+	-	-	-	-
10208*	-	-	-	-	-	-	-	-	-	-	NT		NT	
10213	NT		NT		NT		NT		NT		NT		NT	

Table 3.4 . Results from CCM analysis of individual exons from 13 individuals from different families with Lesch-Nyhan syndrome. An asteris* indicates that only maternal DNA was available for analysis. (-) indicates no cleavage product (+) indicates a cleavage product . (?) indicates a possible cleavage product where interpretation was difficult and the results not clear. (NT) indicates that the exon was not tested.

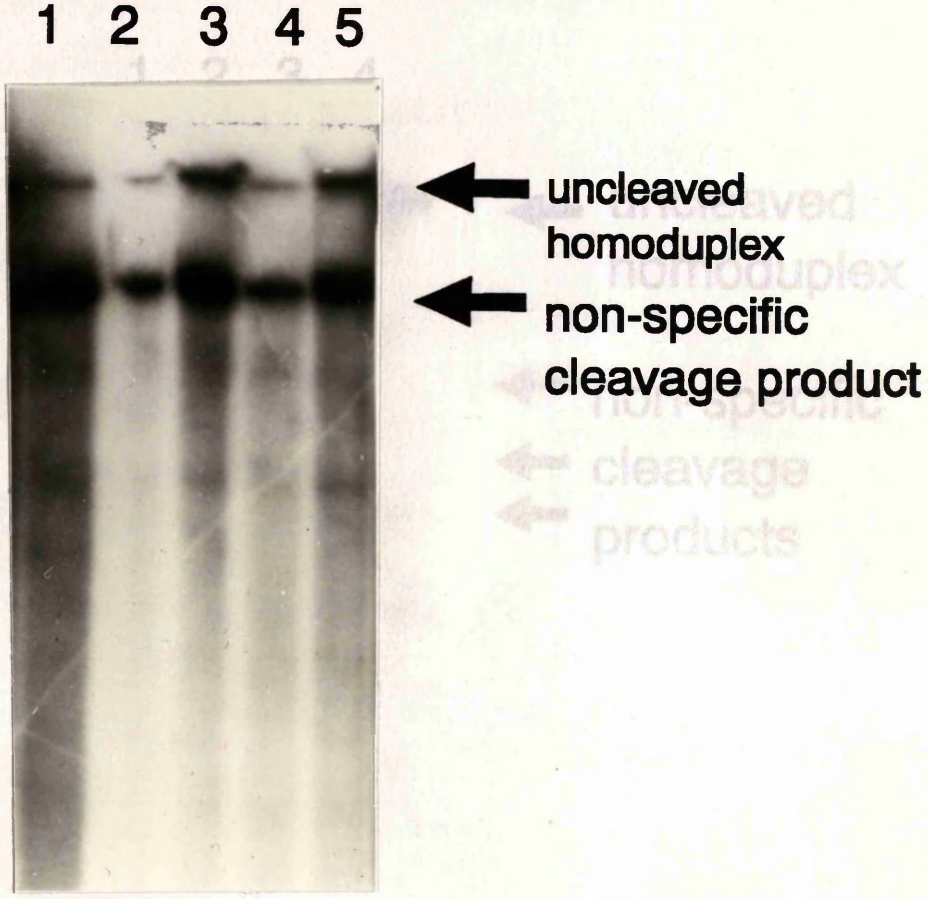


Figure 3.15: Autoradiograph of CCM analysis of exon 7 of the *HGPRT* gene showing a non-specific cleavage product in all lanes. Lanes 1-4 are family members from the pedigree HPRT North Mymms and lane 5 is a control sample.

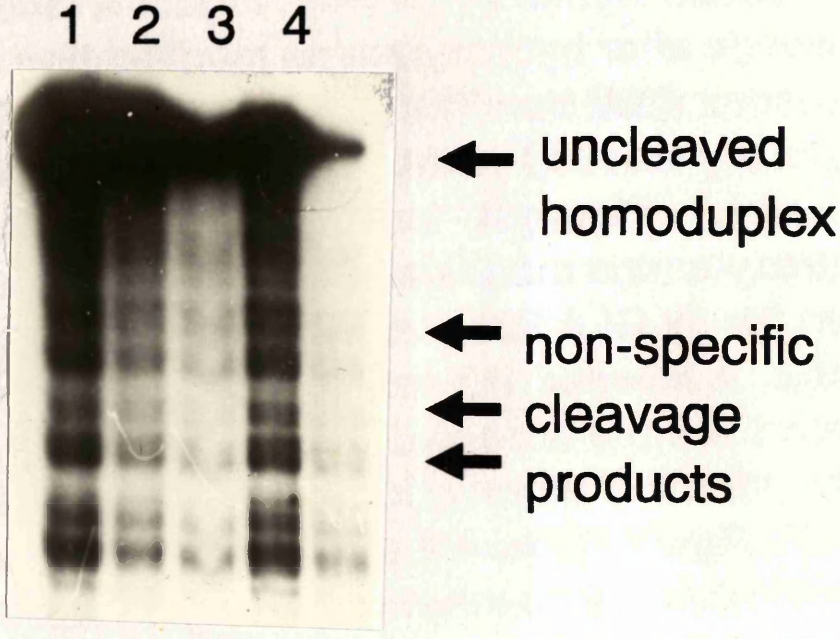
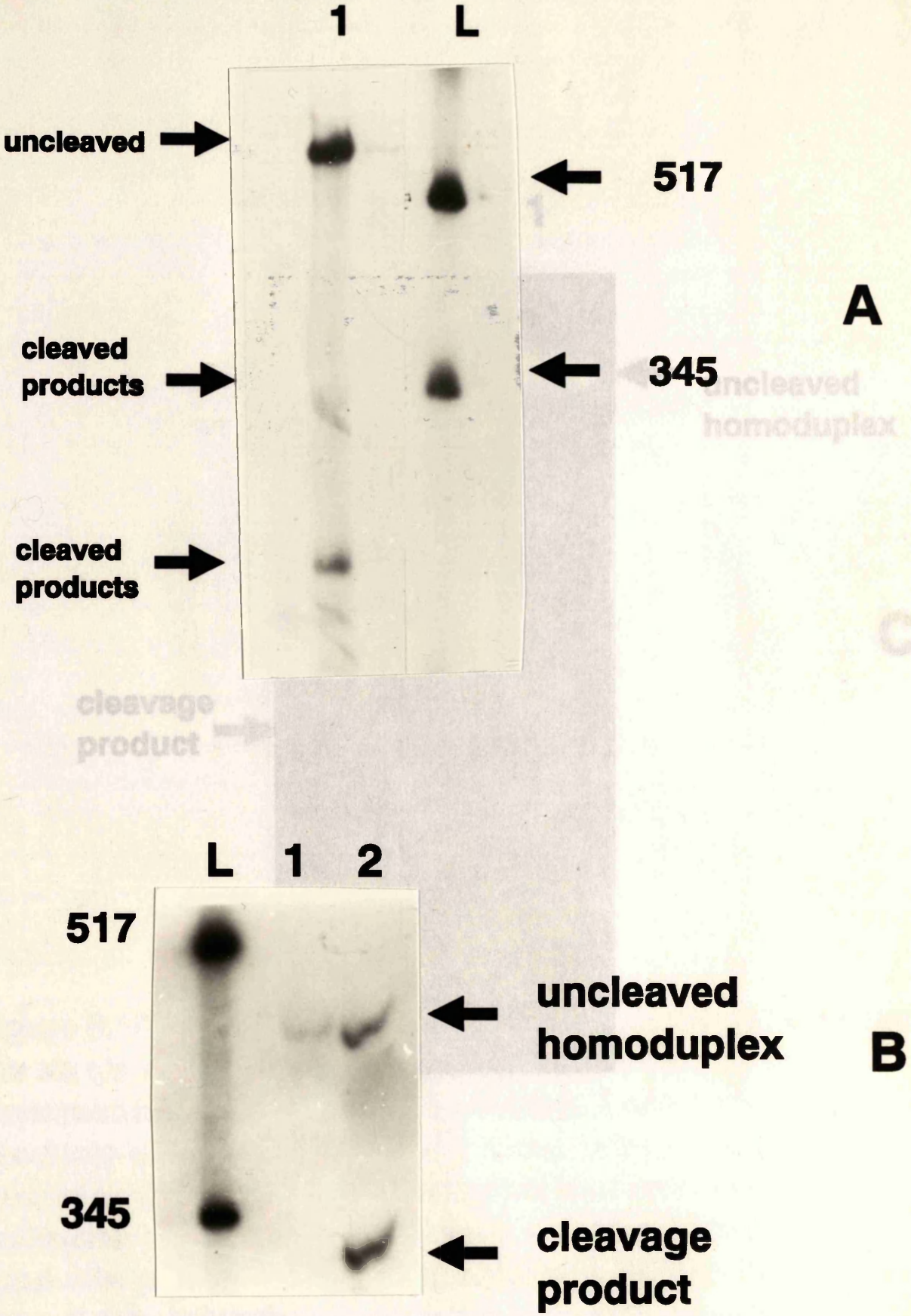
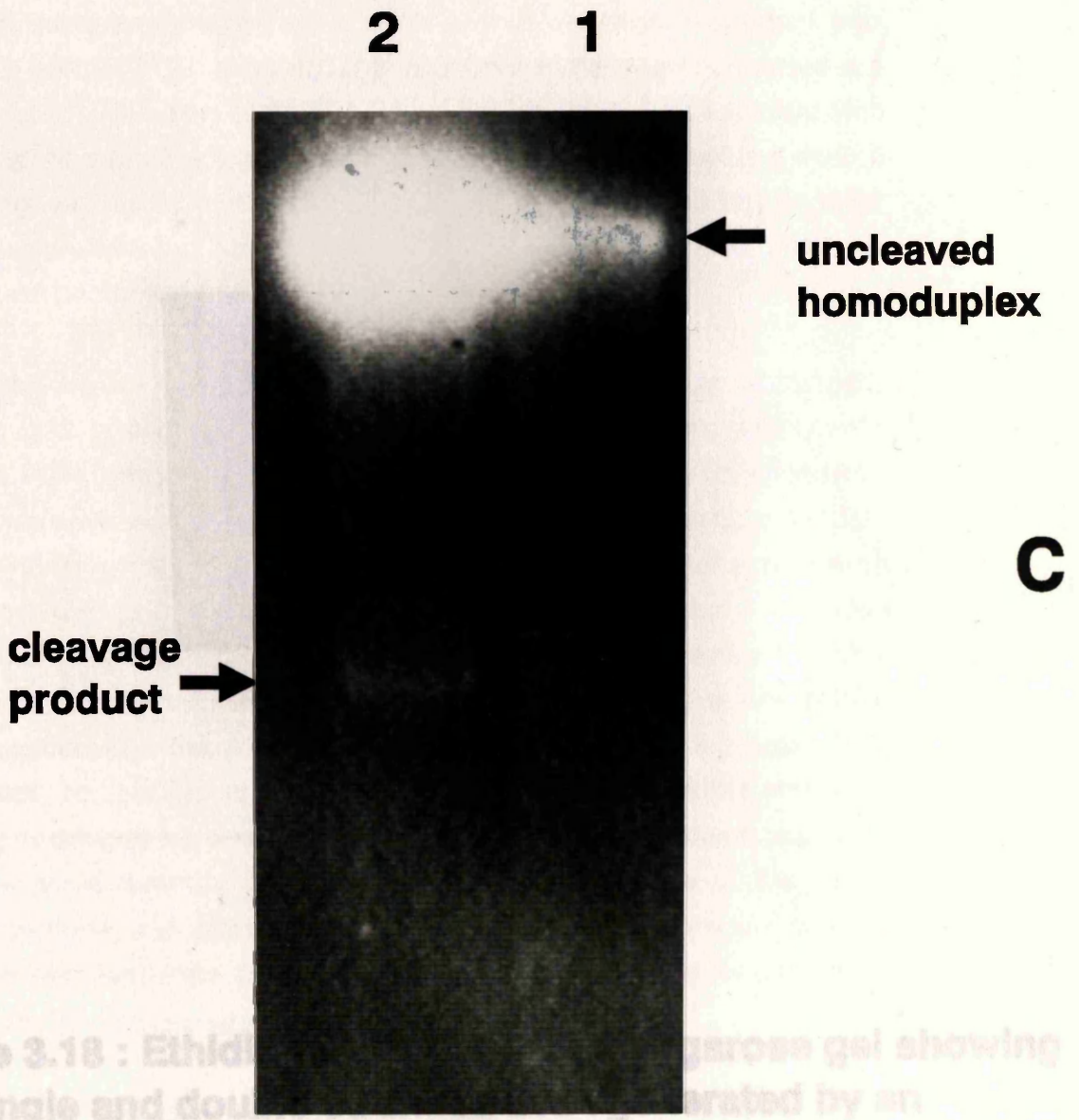


Figure 3.16: Autoradiograph of CCM analysis of exon 3 of the *HGPRT* gene . Lane 1 shows the analysis of exon 3 of the index case from the pedigree HPRT Codicote while lanes 2-4 show the cleavage of exon 3 from normal individuals. The non-specific cleavage products are indicated by arrows.

Figure 3.17 : Autoradiograph of CCM analysis showing cleavage in three exons in three individuals from families with Lesch-Nyhan syndrome. Figure A lane 1 shows cleavage after hydroxylamine modification of exon 2 of the index case from the family Gla 7292 annealed to an internally labelled probe. A labelled 1kb ladder was run in lane L. Figure B, lane 2 shows cleavage following hydroxylamine modification of exon 6 of the index case from family GLA 7291 annealed to an end-labelled probe. A labelled 1kb ladder was run in lane L and a control sample annealed to the same probe and treated in an identical manner to the index case DNA, is run in lane 1. Figure C, lane 2 shows the cleavage following modification by osmium tetroxide of exon 5 from the mother of the index case from the pedigree 10195 after annealing to an end-labelled probe. A control DNA sample annealed to the same probe and treated identically to the mothers sample, is shown in lane 1.


1
L
uncleaved
517
cleaved products
345
cleaved products
A
L
1
2
517
345
uncleaved homoduplex
cleavage product
B


2
1
uncleaved
homoduplex
cleavage
product
C

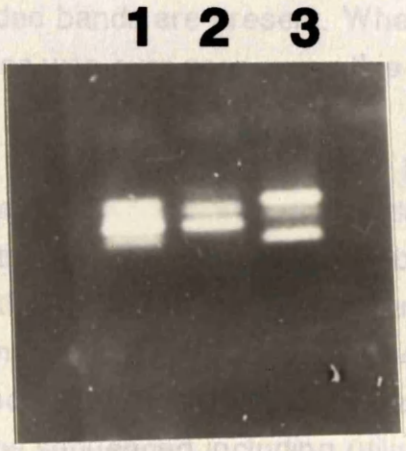


Figure 3.18 : Ethidium bromide stained agarose gel showing the single and double-stranded DNA generated by an asymmetric PCR using exon 2 primers. Lane 1 shows the products obtained when exon 2 sense primers were in excess and lane 2, the products obtained when exon 2 antisense primers were in excess. Lane 3 shows the products generated from an asymmetric PCR using exon 2 primers with the antisense primer in excess and with a higher ratio of antisense to sense primer.

1/150, 1/200 were used. Secondly, the DNA from the original PCR reaction used as a template for the asymmetric PCR was used in a normal PCR reaction to ensure that the DNA was suitable to allow amplification. Figure 3.19. shows the results of these modifications with the asymmetric PCR for exon 7. No bands single or double-stranded were seen after asymmetric amplification using any primer ratios, while a double-stranded product was seen when a normal PCR using equal primer concentrations was carried out using the eluted DNA as a template. A similar situation was apparent with all the attempted exon 3 asymmetric PCR's. When primers amplifing exon 5 were used for asymmetric PCR a smear is consistently seen but no clear single or double-stranded bands are present. When used as a template for sequencing no sequence was ever seen using this product.

For the exons where a single-stranded product was obtained , sequencing was primarily attempted using the same primers which were used for the PCR reaction. Sequence data was obtained only for exon two . Internal primers were then designed (Table 2.1) and some sequence data was obtained for exons 6 and 4. Where an exon would not produce an asymmetric PCR product, other strategies were attempted to obtain products which could be sequenced including utilisation of double-stranded PCR products as a sequencing template and phosphorylation of one primer in a PCR reaction with the product then being digested with a phosphatase in an attempt to obtain a single-stranded product. Double-stranded sequencing produced no sequence data while the phosphatase approach produced a small quantity of very poor sequence in some of the exons tested. Formamide was also used in the sequencing reactions but this did not improve the sequence obtained. An asymmetric product could not be obtained from the double-stranded cDNA from pedigree10427.

Sequence changes were found in 3 pedigrees. The index case from the pedigree 7291 had a 1 bp deletion in exon 6, namely a T deleted at position 462 (figure 3.20) which would result in a premature termination of transcription in exon 7 and therfore the production of a truncated protein. There was no DNA remaining for clarification of this mutation by automated sequencing and the manual sequence is shown . Sequence could only be obtained for one strand of the exon. However a heteroduplex band was clearly seen in exon 6 of the index case and the position of the deletion is consistant with the size of the cleavage product after CCM analysis. The

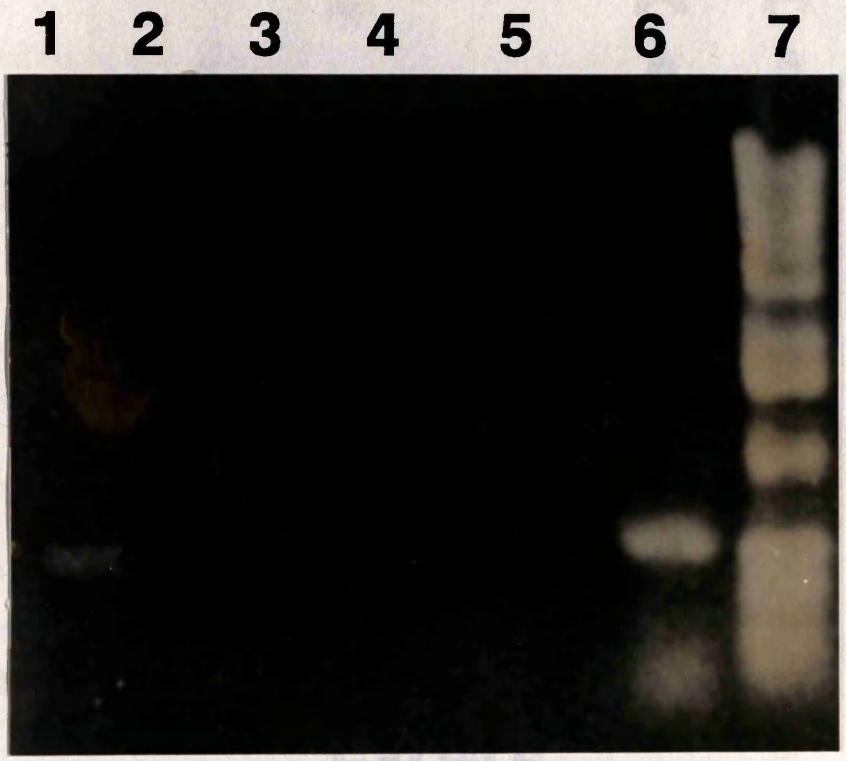
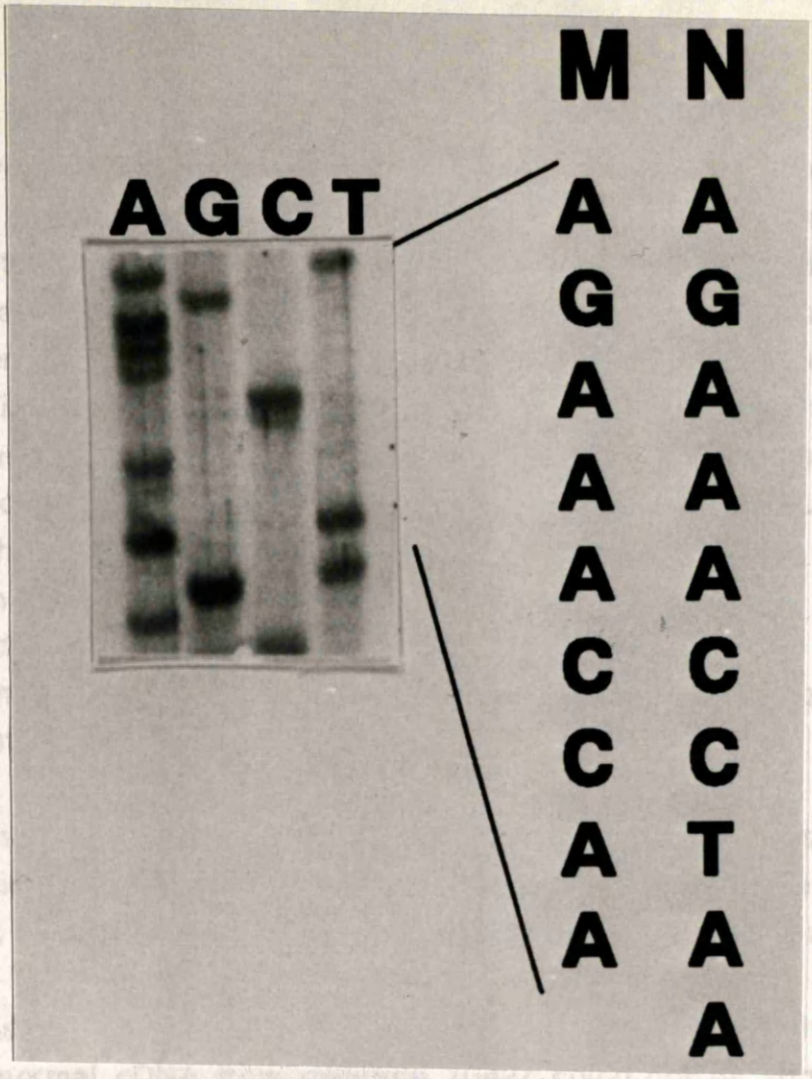


Figure 3.19 : Ethidium bromide stained agarose gel showing the products of a symmetric and asymmetric PCR amplification using exon 7 primers. Lane 1 shows an aliquot of the eluted template DNA run out on the gel, lanes 2-5 show different ratios of the excess to limiting primers from 1:25 to 1:100. Lane 6 shows the product of a symmetric PCR using exon 7 primers with the template shown in lane 1 used as the template for this reaction. A 1kb ladder is shown in lane 7.



EXON 6 EXON 7

NORM: DIIDTGKTMQTLLSLVRQYNPKMVKVASLLVKRTPR

AFFECT: DIIDTGKTMQTLLSLVRQYNQRWSRSQACW*KGP

Figure 3.20 : Autoradiograph of part of a gel showing the manual sequence of the sense strand of exon 6 from the index case with the mutation HGPRT Gla 7291. The gel shows a deletion of a T nucleotide at position 462 of the *HGPRT* gene. The normal sequence is given in column N, the mutant sequence in column M. The altered protein sequence showing the premature stop codon is also shown.

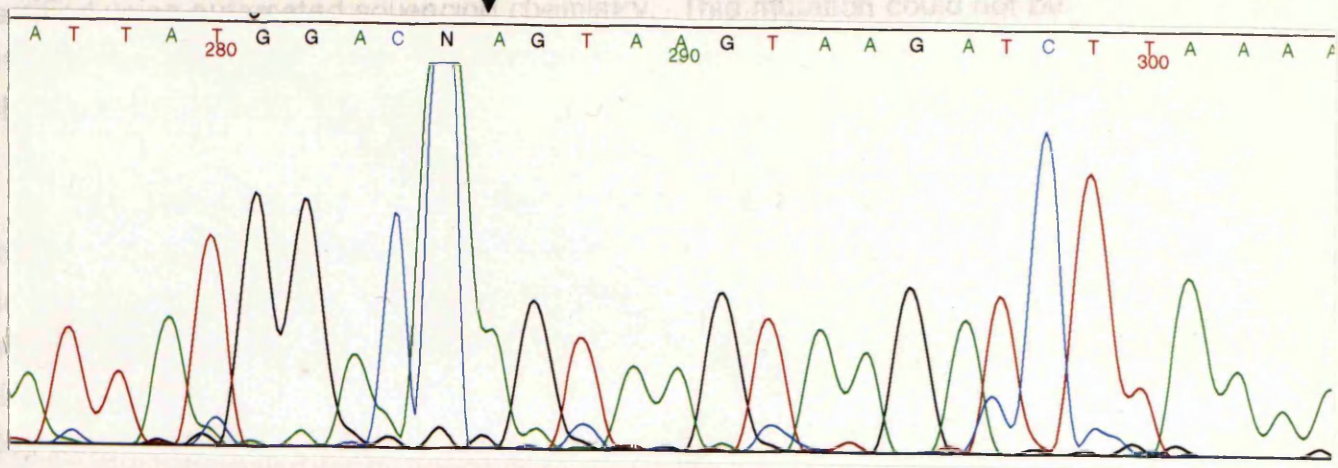
index case from pedigree 7292 had a single G to A nucleotide substitution at position 134 in exon 2 which would result in a substitution of a lysine for an arginine at this position. This mutation was also sequenced using the automated sequencer which gave a better resolution of the mutation originally identified by manual sequencing. The sequence of the index case is shown in figure 3.21. Sequence information was available for both strands and confirmed the mutation. This mutation although not resulting in a truncated protein or a change in the charge of the amino acid, results in a change in the last coding base of exon 2. This may lead to a splicing abberation in the RNA, but no RNA was available from this family to confirm this by the identification of an altered cDNA. The change is unlikely to be a polymorphism as it was not present in any of the other families sequenced but is present in other female family members.

For the pedigree 7293 where a larger exon 4 product was seen, despite obtaining an asymmetric product and obtaining a sequence from a control DNA sample, sequence data could not be obtained using manual sequencing. This was due to the sequence of the exon involving runs of identical bases which thwarted the sequencing chemistry. Further, the larger than normal cDNA from pedigree 10427 could not be sequenced manually as an asymmertic product could not be obtained for sequencing.

3.1.11 Optimisation of Automated Sequencing Chemistry and Mutations Found in the *HGPRT* Gene.

The automated sequencing chemistry involving cycle sequencing was optimised for the *HGPRT* gene using the HGPRT GLA 7292 mutation in the index case as a control. This exon sequenced well and the mutation was clearly identified (figure 3.21). The mother of the index case, a heterozygote was then sequenced and the hererozygote peak was identified . The samples where a mutation had been indicated but could not be identified were then sequenced using the same primers as were used for PCR. The insertion in exon 4 of the index case from the pedigree 7293 could not be sequenced in either direction using this chemistry due to the prevalence of strings of identical bases in the exon. The mother of the index case from pedigree 10195 which showed a heteroduplex band in exon 5 was sequenced. However this exon did not produce a clean sequence and a real double peak could not be identified from amplified background noise

A/G substitution



A

sense normal sequence : TATGGACAGGTAAG
sense mutant sequence : TATGGACAAGTAAG
heterozygote : A/G

B

Figure 3.21 : Part of a chromatogram from an automated sequencer showing the mutation in exon 2 from the Index case from the family Gla 7292. The sequence of the sense strand is shown in A with the position of the A/G substitution indicated. The mutant and normal sequences are shown in figure B.

At nucleotide N at base position 285, the double peak(A/C) ,is due to the sequencing chemistry and is often seen in sequences and is read as a chemistry artefact.

which resulted due to the poor quality of the template. However it did appear that no insertions or deletions were present as these can clearly be identified using automated squencing chemistry. This mutation could not be further characterised and the possibility that this change was a polymorphism could not be excluded.

The index case from pedigree 10427 showed abnormal PCR amplification patterns in exon 7. cDNA was available for the index case and this was shown to be approximately 60bp larger than the normal cDNA. This was sequenced using the automated sequencer chemistry using internal primers close to exon 7. The sequence shown in figure 3.22 contained a 60bp addition. When this sequence was compared to a sequence database it was shown to be an alu repeat which had been inserted between exons 6 and 7. As this region could not be sequenced from the DNA due to the amplification problems, the exact position of the insertion in the DNA or the possible splice site alterations could not be pinpointed. However, no abnormalities were seen in exon 6 or the following intronic regions so it is most likely that the abnormality occured in the region of exon 7. This change is very significant and the large size of the insertion will obviously lead to a truncated protein and probably alters the entire folded conformation of the protein. DNA was not available for automated sequencing from other pedigrees.

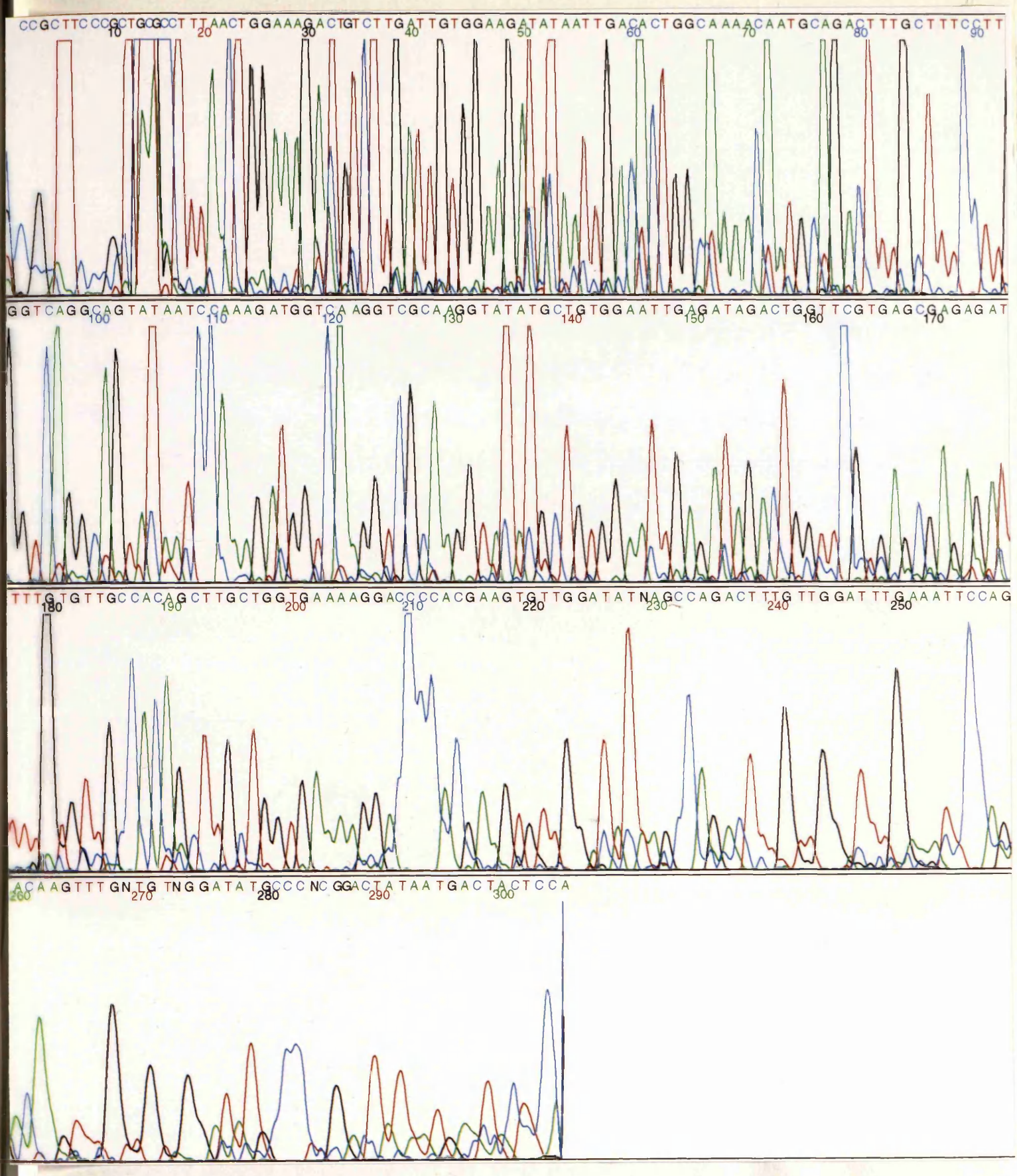
3.1.12 Carrier Detection in the *HGPRT* Gene.

3.1.12. 1 Linkage Analysis Using a Tetranucleotide Repeat.

A PCR based strategy was used for linkage analysis in 9 pedigrees where DNA was available from key family relatives. Edwards et al., (1991) reported the existence of a tetranucleotide short tandem repeat located within the *HGPRT* gene and oligonucleotide primers were manufactured which were complementary to the sequences flanking this repeat. The primers were 5'-end labelled using γ-ATP and PCR carried out according to the conditions described in 2.12 before the allele length were resolved on a denaturing polyacrylamide gel.

The PCR reaction was very efficient and product bands of the correct size were identified by comparison of the products to a labelled 1kb ladder.

Figure 3.22 : Part of a chromatogram from an automated sequencer showing the sequence of the sense strand of the cDNA from the Index case from the pedigree 10427. Figure A shows the chromatogram with the insertion begining at base 130. Figure B shows the sequence of the normal and mutant cDNA with the insertion being highlighted in italics. Figure C shows the position of the insertion into the cDNA of the *HGPRT* gene in this family.

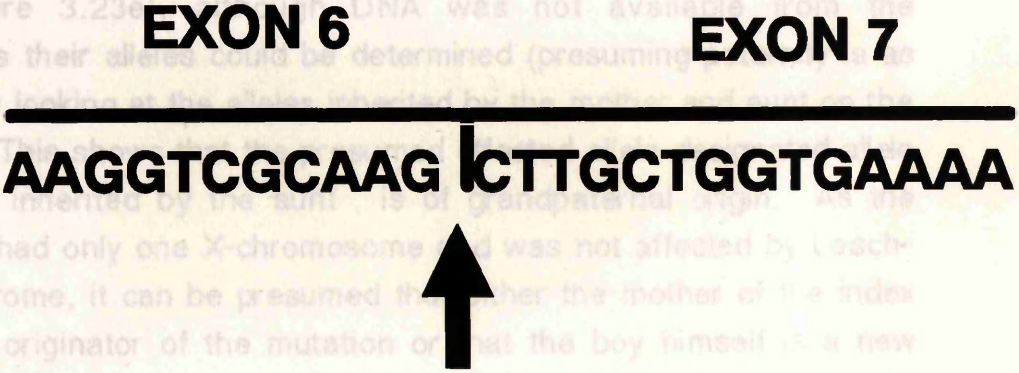

CCGCTTCCCGCTGCGCCTTTAACTGGAAAGACTGTCTTGATTGTGGAAGATATAATTGACACTGGCAAAACAATGCAGACTTTGCTTTCCTT
10 20 30 40 50 60 70 80 90
GGTCAGGCAGTATAATCCAAAGATGGTCAAGGTCGCAAGGTATATGCTGTGGAATTGAGATAGACTGGTTCGTGAGCGAGAGAT
100 110 120 130 140 150 160 170
TTTGTGTTGCCACAGCTTGCTGGTGAAAAGGACCCCACGAAGTGTTGGATATNAGCCAGACTTTGTTGGATTTGAAATTCCAG
180 190 200 210 220 230 240 250
ACAAGTTTGNTGTNGGATATGCCCCNCGGACTATAATGACTACTCCA
260 270 280 290 300

A

Normal cDNA : GGTCGCAAGCTTGCTGGTGAAAAG

Mutant cDNA : GGTCGCAAG*GTATATGCTGTGGAA*
TTGAGATAGACTGGTTCGTGAGCG
***AGAGATTTTGTGTTGCCACAG*CTTG**
CTGGTGAAAAG

B



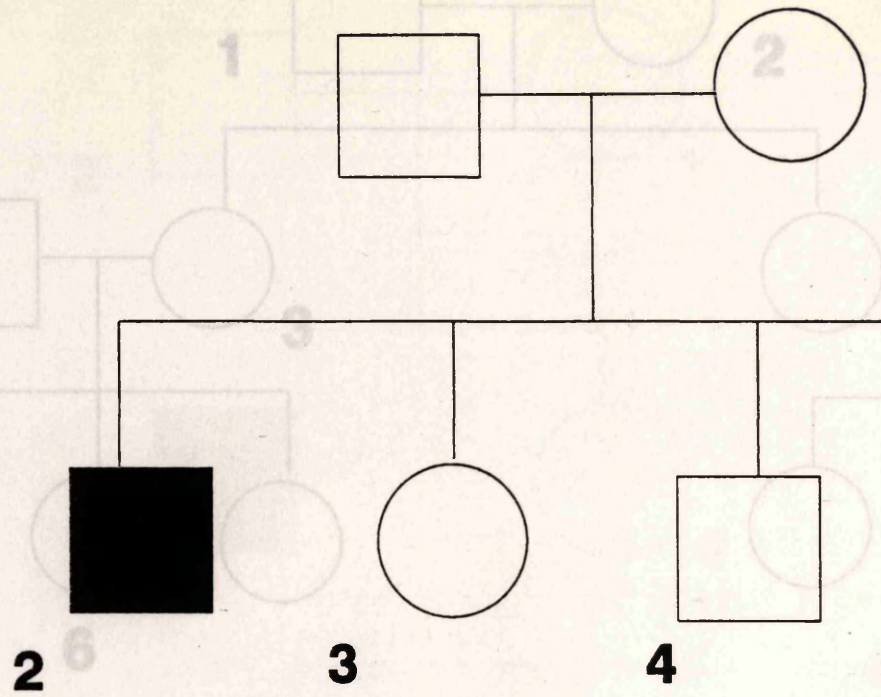
C

No non-specific product bands were seen. The gels were run for varying times to obtain optimum separation of the alleles and the majority of alleles could be separated by running the gels at 2000 Volts for 6-8 hours. Autoradiography was carried out for varying lengths of time depending on the intensity of the signal obtained after 2 hours. Three different PCR reactions were carried out and at least three different gels run for each linkage analysis before results were reported.

DNA was available from the necessary relatives in 9 families with an index case with Lesch-Nyhan syndrome. The 9 families were the pedigree numbers: 10202, 10189, 7293, 10188,10185,10196, 10198 , 7292 and 10195. The pedigrees of eight of the families and the autoradiographs showing the alleles amplified from the family members are shown in figures 3.23 a-h. At this stage in the project it was not known which of the index cases were affected as the result of a new mutation.

In families 10202, 10189, 7293, 10188 (figures 3.23.a,.b,.c and .d respectively), the mothers are informative and female relatives who have inherited the presumed affected allele can be clearly identified. In families 10185 and 10196 (figures 3.23e and 3.23f), the grandpaternal and grandmaternal genes help to establish the inheritance pattern to the aunts of the index cases and allows further clarification of their carrier status. In family 10185 (figure 3.23e), although DNA was not available from the grandparents their alleles could be determined (presuming paternity is as expected) by looking at the alleles inherited by the mother and aunt on the index case. This shows that the presumed affected allele designated allele A, although inherited by the aunt , is of grandpaternal origin. As the grandfather had only one X-chromosome and was not affected by Lesch-Nyhan syndrome, it can be presumed that either the mother of the index case is the originator of the mutation or that the boy himself is a new mutation, indicating that the aunt is not a carrier unless the grandfather was a gonadal mosaic, which is a less likely possibility. The DNA was available from the grandparents of the family 10196 (figure 3.23 f) . Again the presumed affected X-chromosome(allele A) is of grandpaternal origin therefore despite having inherited the allele which cosegregates with the disease the aunt is probably not a carrier, barring the possibility of gonadal mosaicism. The sisters also appear not to be carriers as they have inherited the B allele which does not segregate with the disease.

3.23 : Autoradiographs showing linkage analysis in eight Lesch-Nyhan families using a tetranucleotide repeat located within the *HGPRT* gene. In each family the pedigree is shown, with the lanes corresponding to the designated number of the family member given in the pedigree. The allele status of the individual is also shown. Figures a-h represent pedigrees 10202,10189,7293, 10188, 10185, 10196, 10198 and 7292 respectively.



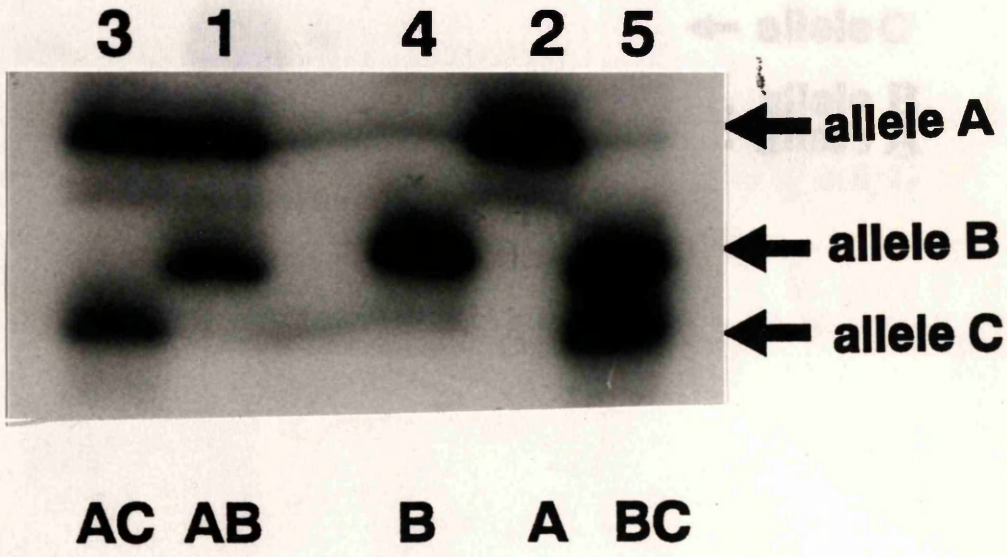
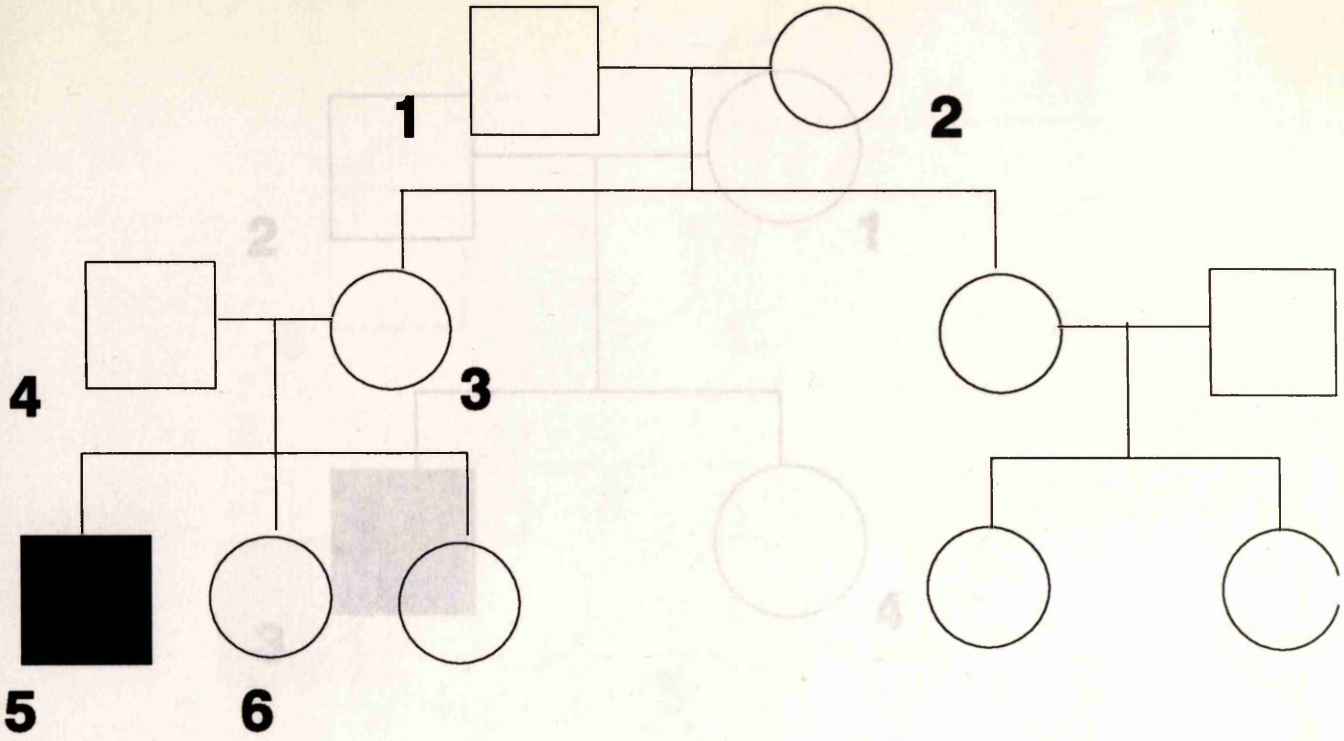


Figure 3.23.a



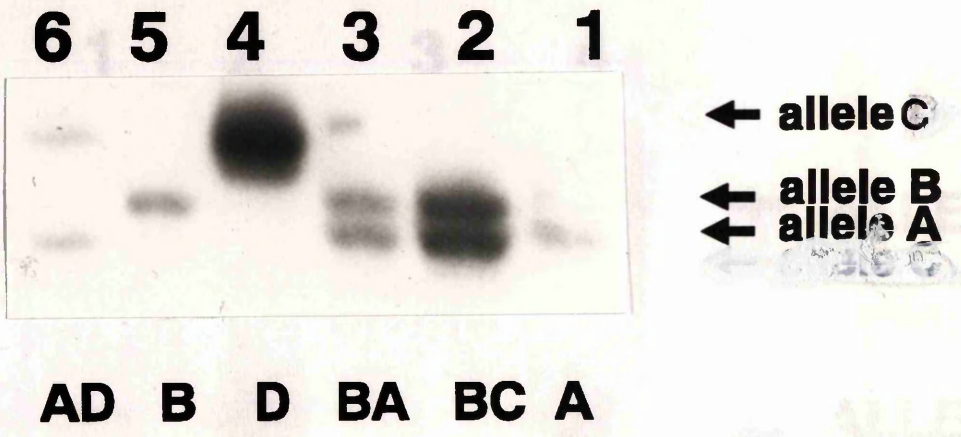
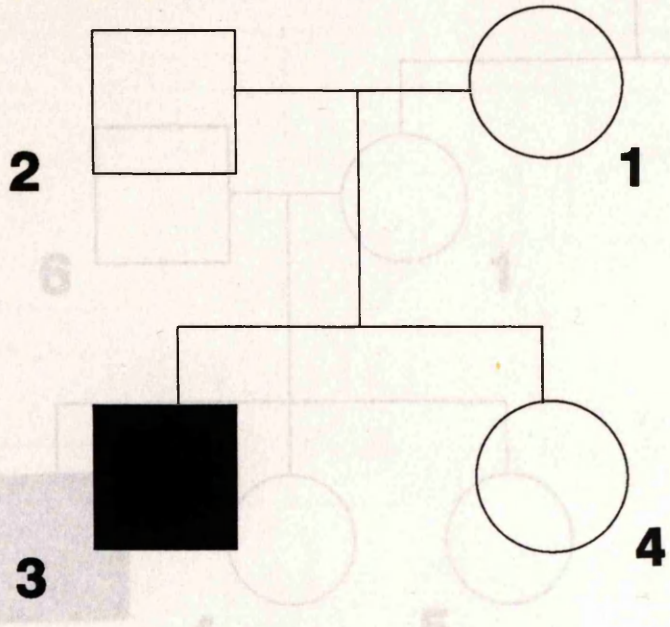


Figure 3.23.b



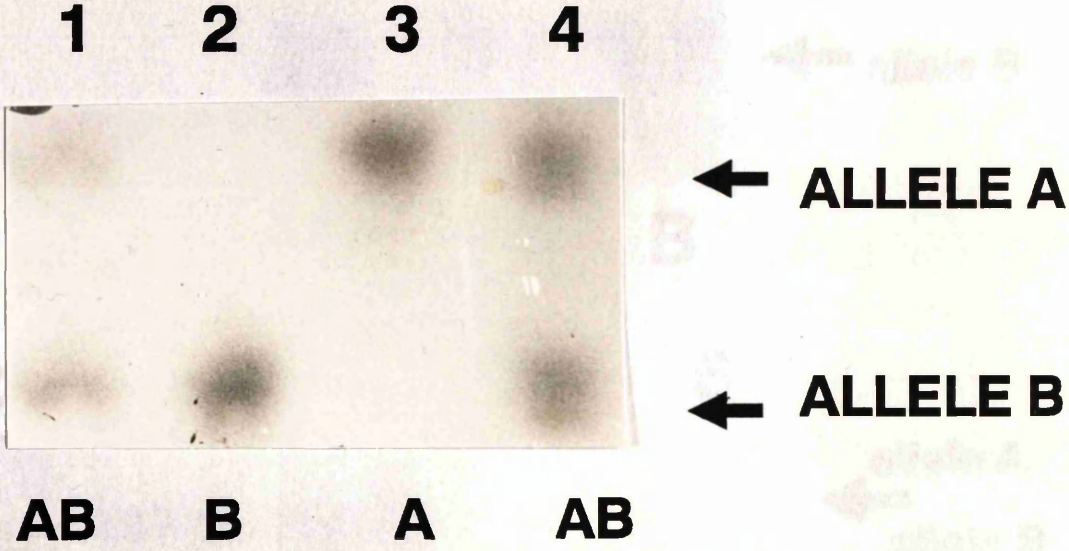


Figure 3.23.c

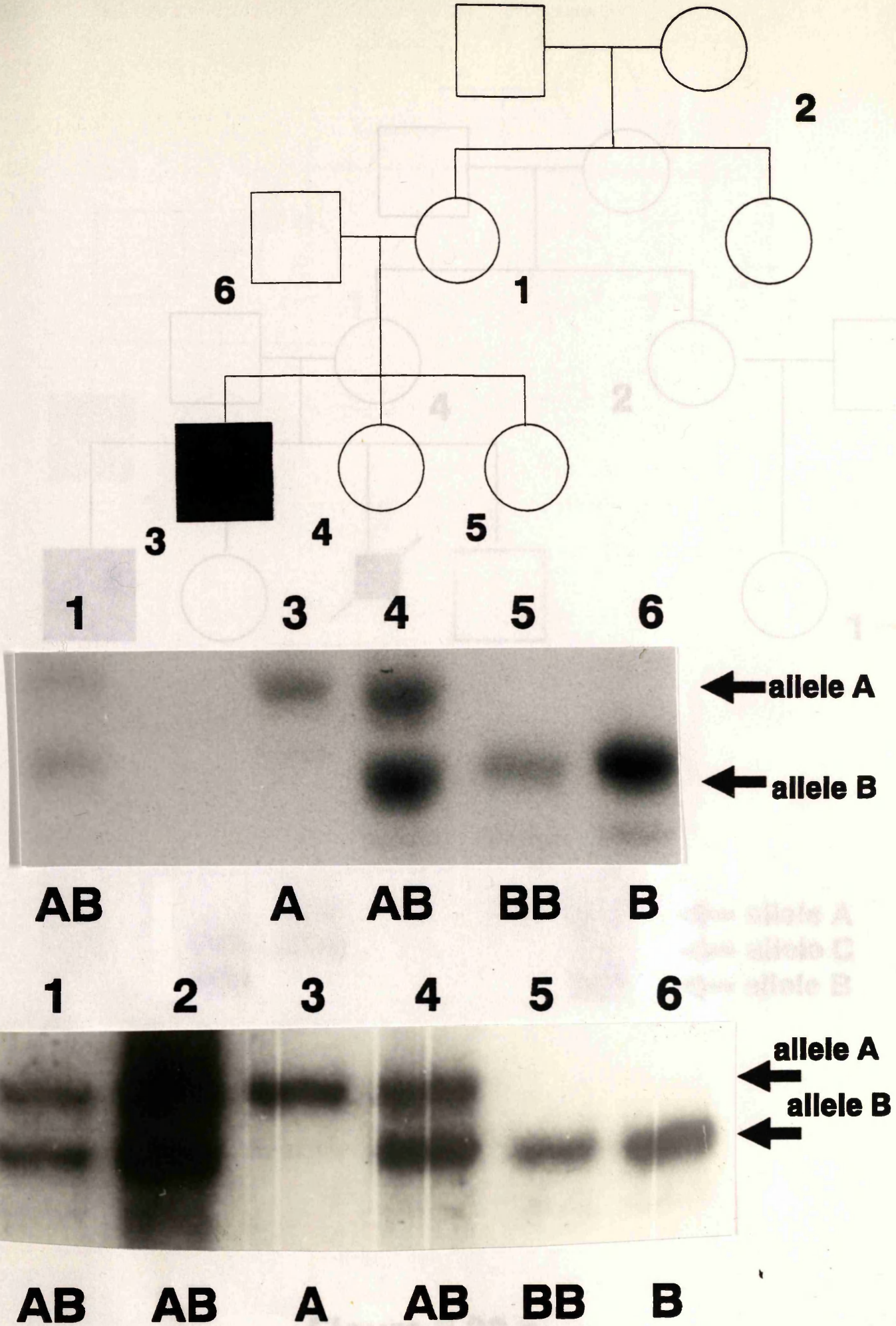

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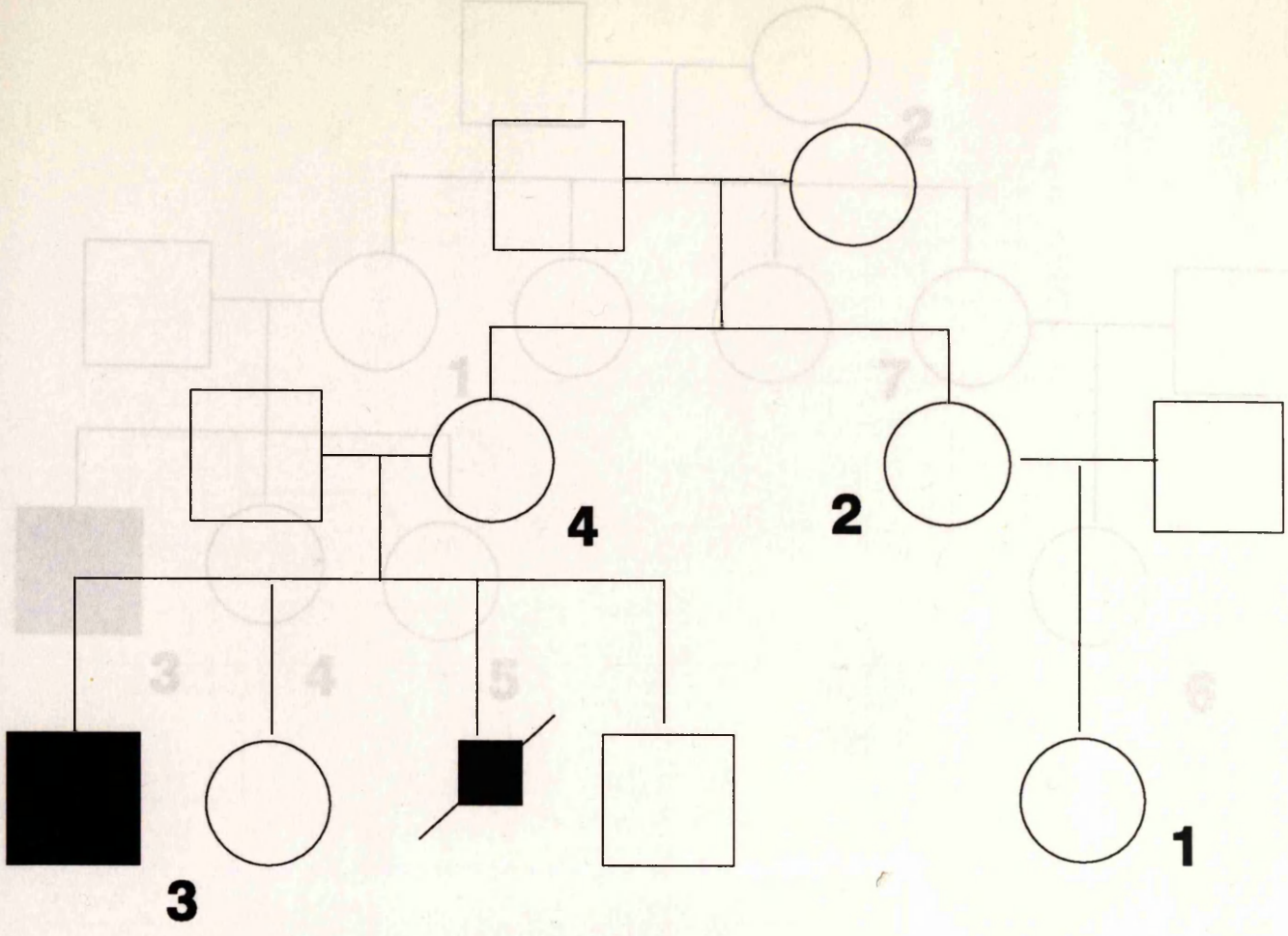
Figure 3.23.d



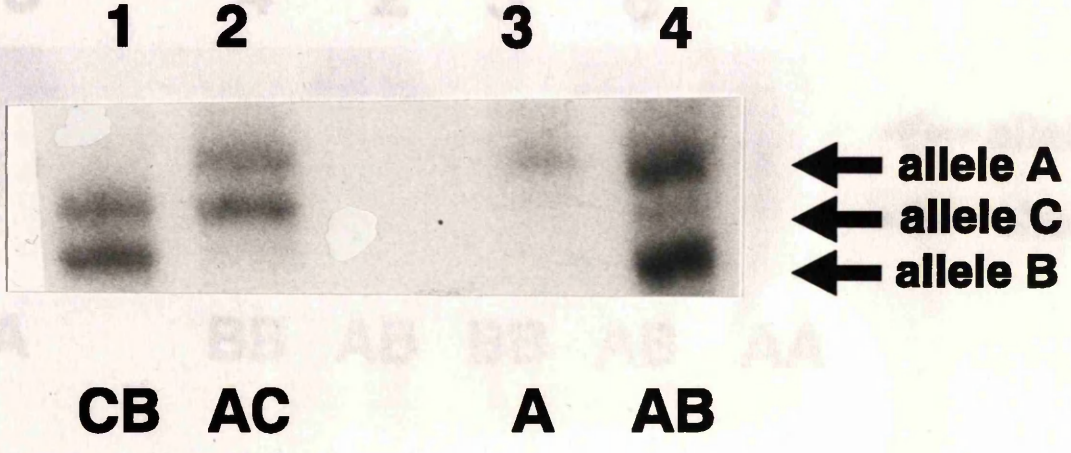
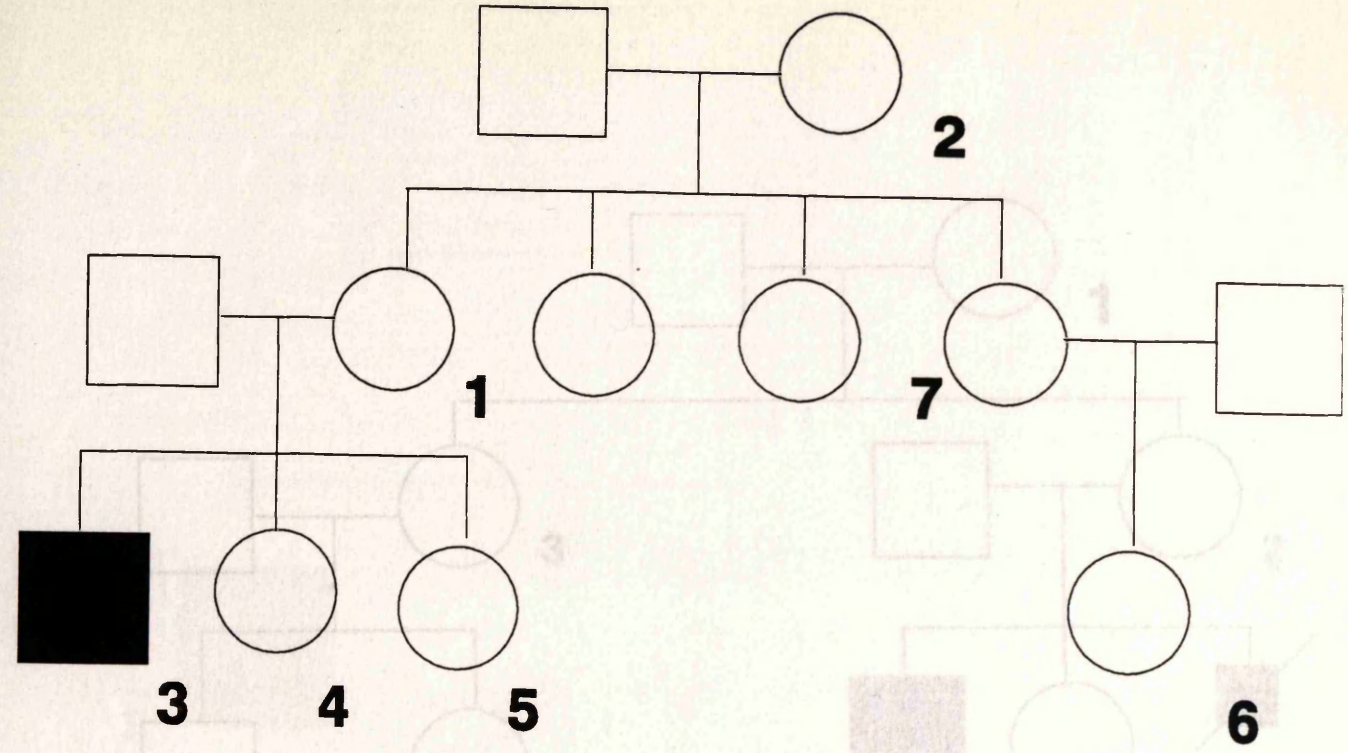


Figure 3.23.e



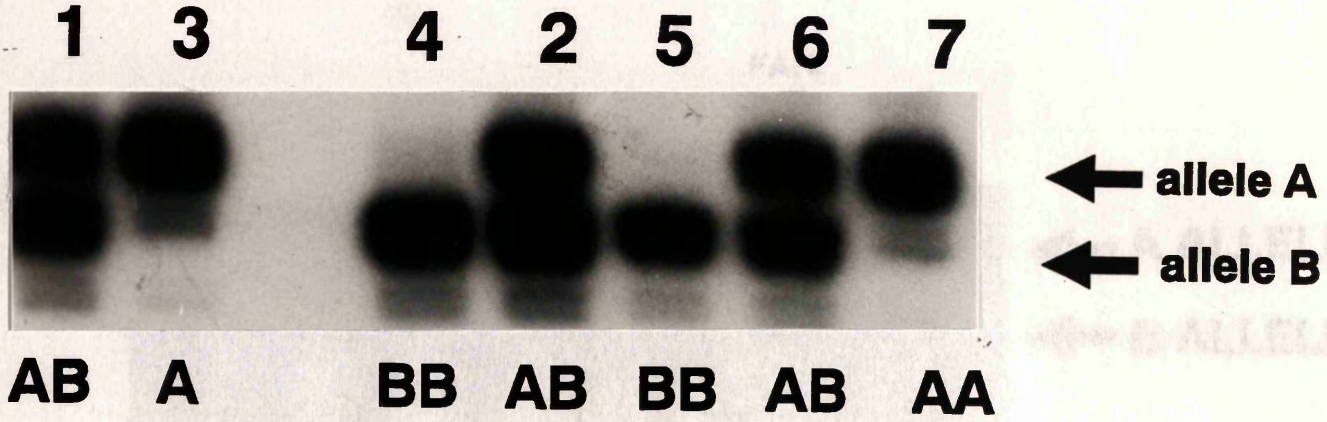
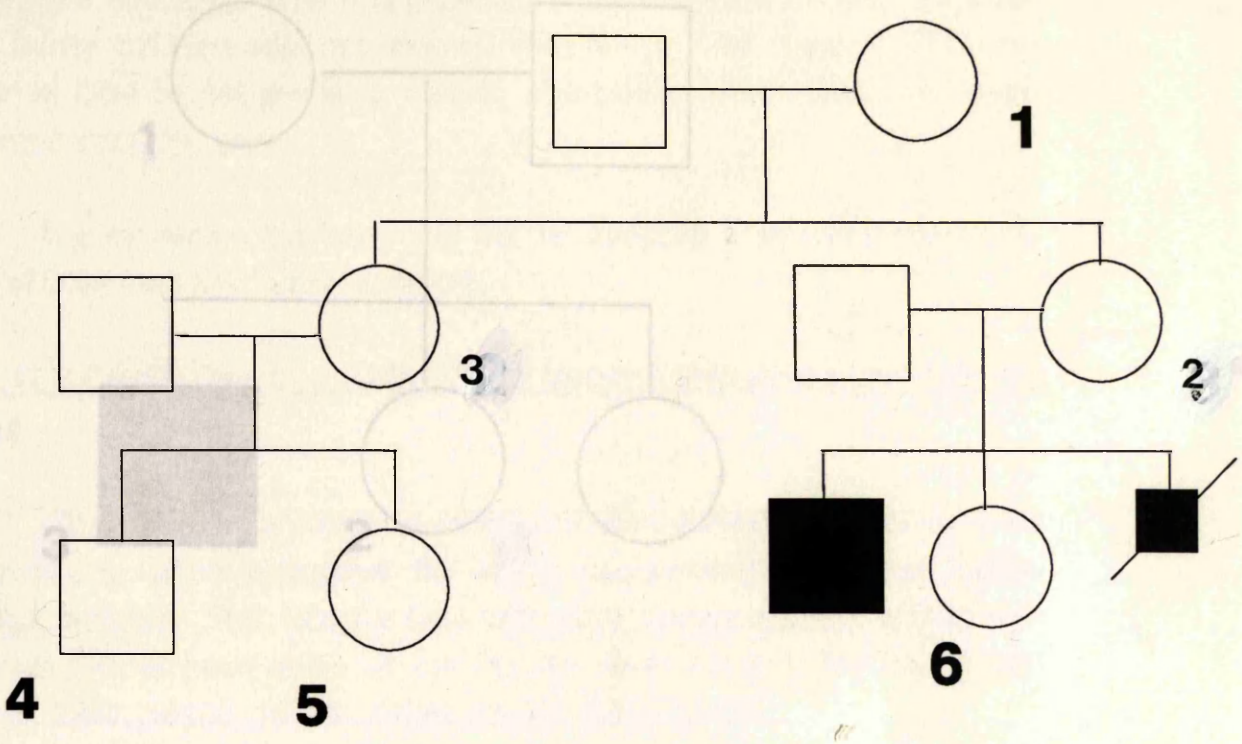


Figure 3.23.f



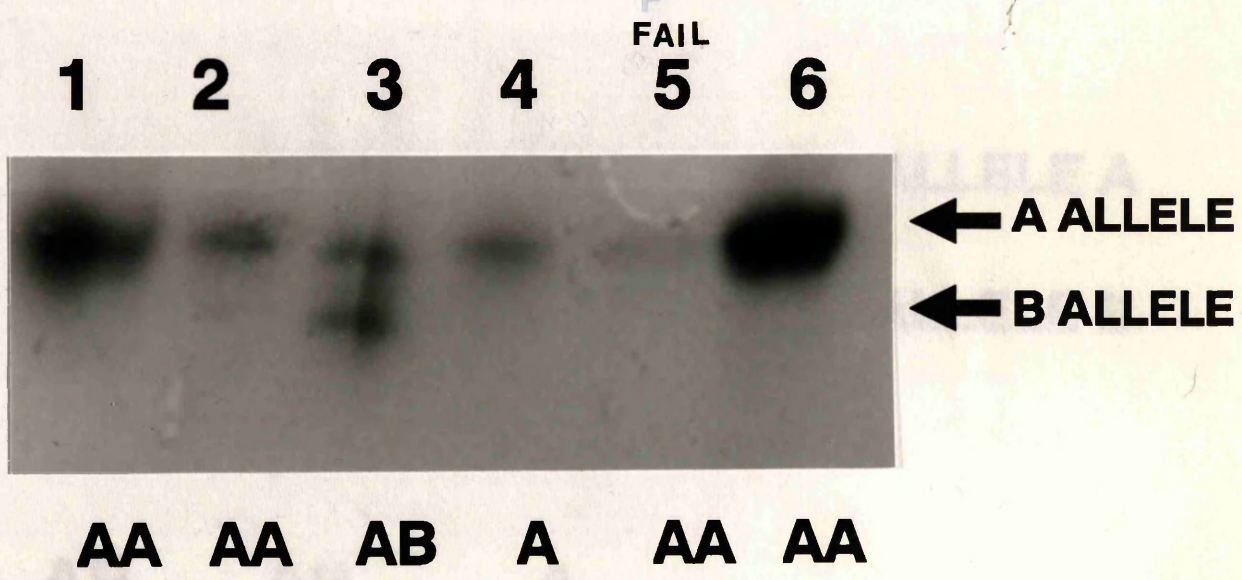
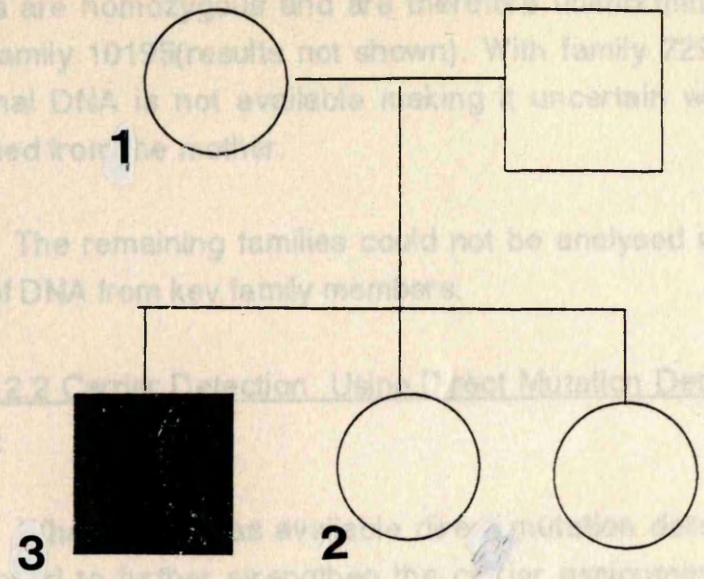


Figure 3.23.g



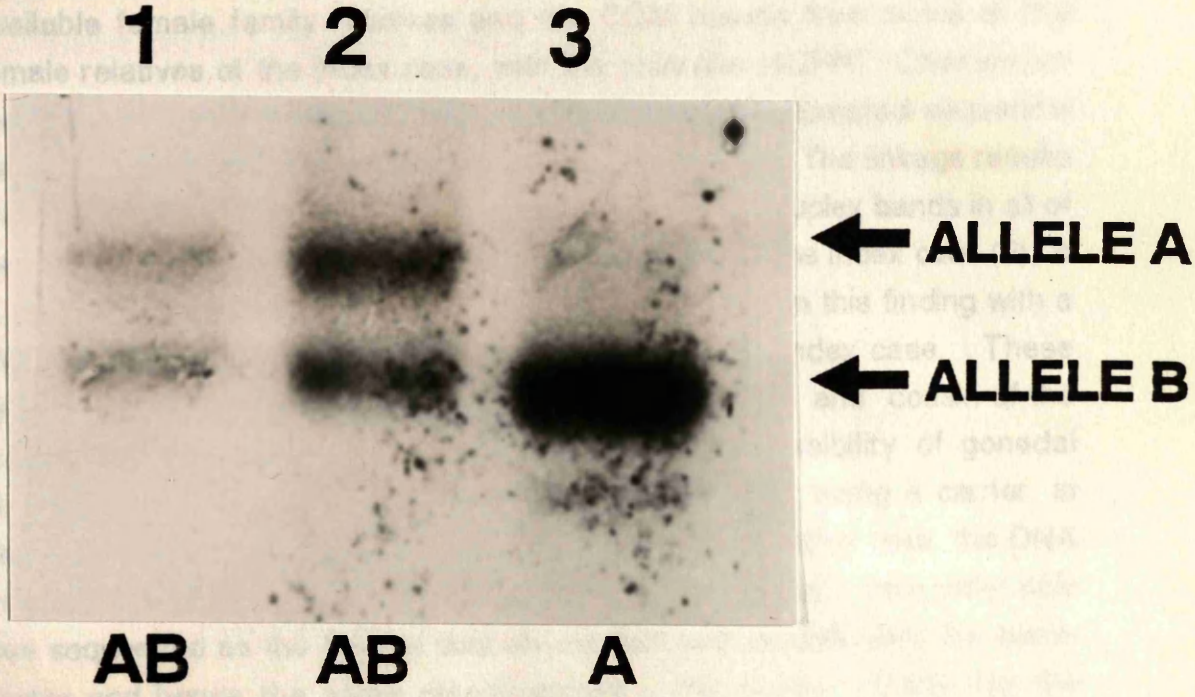


Figure 3.23.h

In family 10198 (figure 3.23.g) the maternal and grandmaternal alleles are homozygous and are therefore uninformative as was the case with family 10195(results not shown). With family 7292 (figure 3.23.h) the paternal DNA is not available making it uncertain which allele has been inherited from the mother.

The remaining families could not be analysed in this way due to the lack of DNA from key family members.

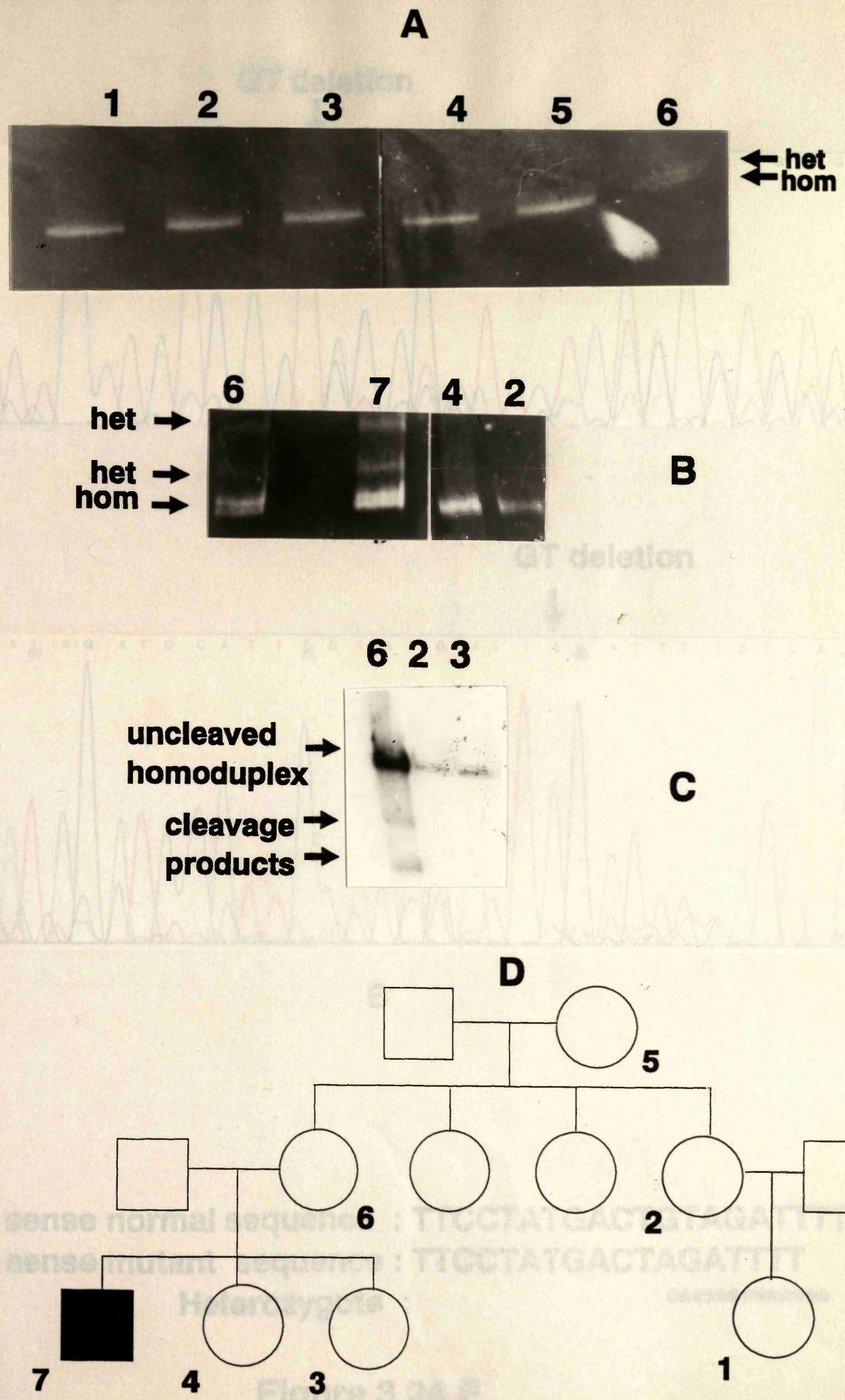
3.1. 12.2 Carrier Detection Using Direct Mutation Detection in the *HGPRT* Gene

Where DNA was available direct mutation detection strategies were employed to further strengthen the carrier assignments designated by the linkage analysis. This was the case with eight pedigrees where DNA was available for at least some of the female relatives including pedigrees: 10196, 7292, 10188, 10195, 10199, 10197, 7293, 10198.

The results from these analyses are shown in figures 3.24-31. Figure 3.24 shows the hydrolink results from a long and short gel from all the available female family relatives and the CCM results from some of the female relatives of the index case, with the mutation HGPRT Cheltenham (pedigree 10196). The sequences generated from the automated sequencer for some key female relatives are also shown in figure E. The linkage results are shown in figure 3.23.f. From the absence of heteroduplex bands in all of the female relatives with the exception of the mother of the index case (6), it is clear that only she is a carrier. The CCM results confirm this finding with a cleavage product only being seen in the mother of the index case. These results support the linkage results and as both the aunt and cousin of the index case inherited the grandpaternal allele, the possibility of gonadal mosaisism in the grandfather resulting in the aunt (2) being a carrier is negligible. To rule out this possiblity and to confirm the other data, the DNA from the mother(6) ,sister (4) and aunt(2), was sequenced . One sister only was sequenced as the linkage data shows that both sisters carry the same alleles and hence the same chromosomes. The mother clearly has the mutation while the others show a normal sequence, supporting the data obtained from the other protocols. The written sequence from the index case and a normal individual is also shown.

Figure 3.24 - 3.31: Carrier detection in females from eight families with Lesch-Nyhan syndrome.

Figure 3.24. : Carrier detection in exon 3 of the family HGPRT Cheltenham. Figure A shows an ETBR stained hydrolink gel with the results of heteroduplex detection analysis on female family members available while figure B, shows a 30cm gel loaded and run with a different PCR reaction using the DNA from the family members. The lane numbers correspond to the numbers beside the individuals in the pedigree and the heteroduplex and homoduplex bands are indicated by arrows. On the longer gels second slower migrating bands are sometimes apparent with this mutation and these are indicated by arrows. Figure C, shows the results of CCM analysis on the mother of the index case (lane 1) and the grandmother (lane 2) and aunt (lane 3) of the index case , after modification of the duplexes formed with osmium tetroxide. The probe was internally labelled and the arrows indicate the cleavage products. A partial family pedigree is shown in figure D. Figure E, shows part of a chromatogram from an automated sequencer, showing the sequence of the sense strand from exon 3 of the mother (A), index case (B), aunt of the index case (C) and sister of the index case (D). The position of the GT deletion is indicated in the index case and mother and the position where the GT deletion would be, is indicated in the other two samples. The normal and mutant sequences are also shown and compared.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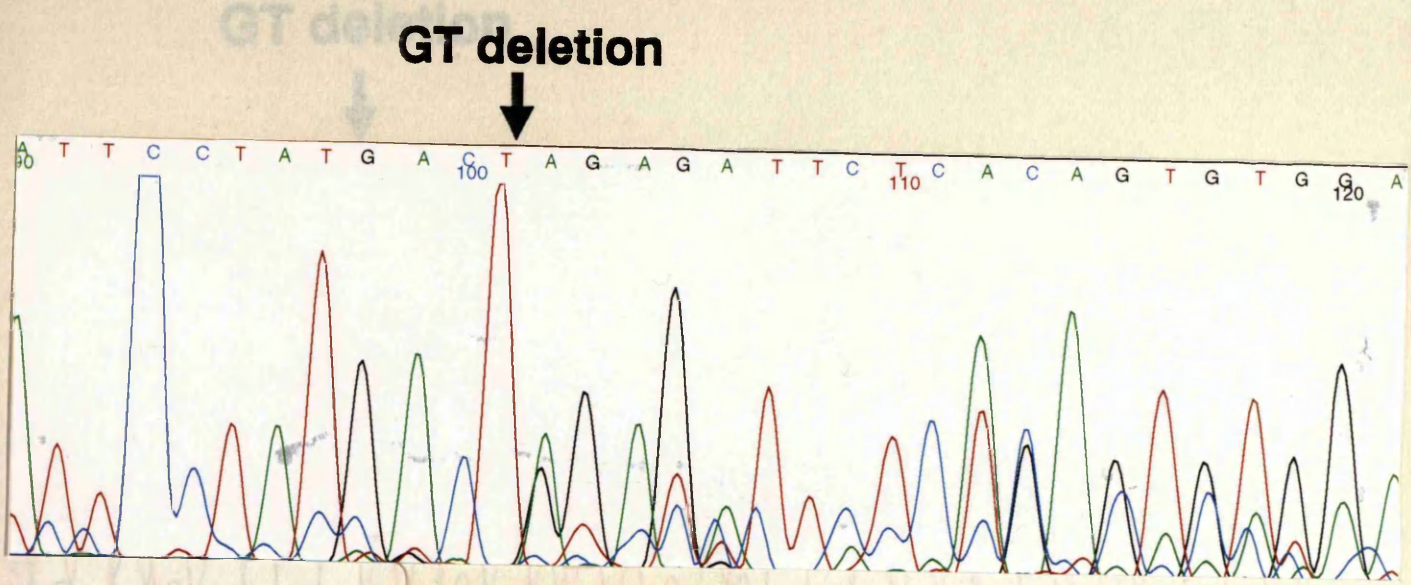
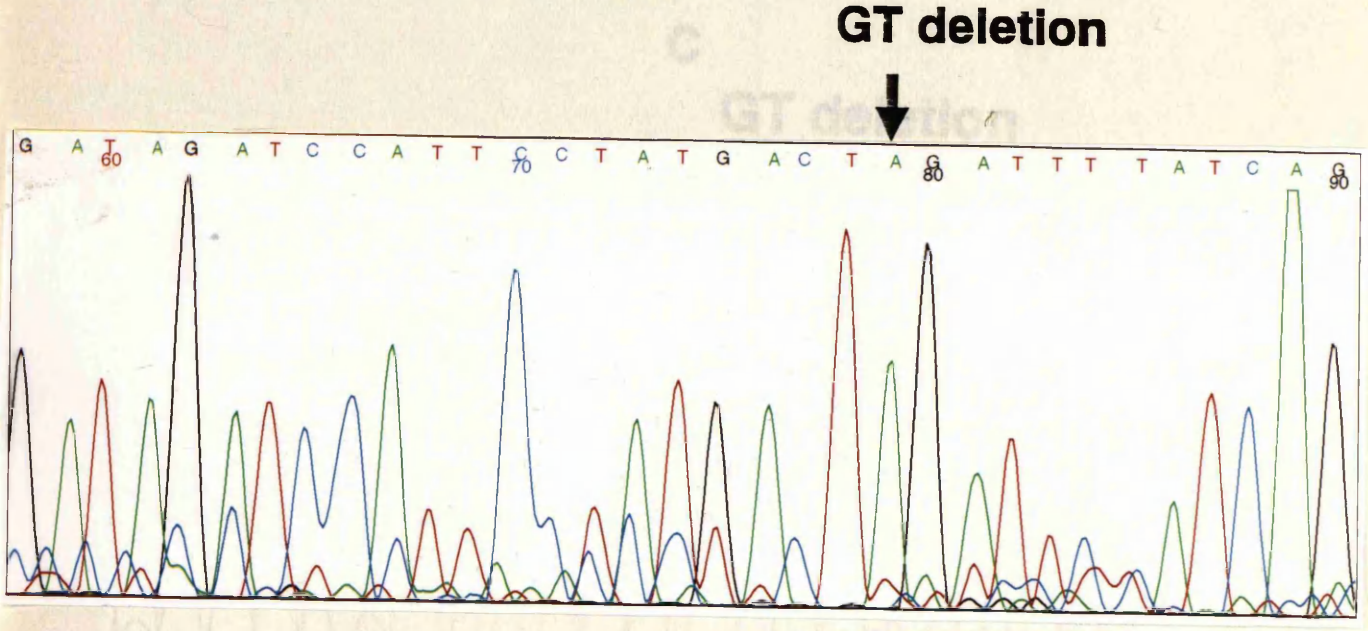


A



B

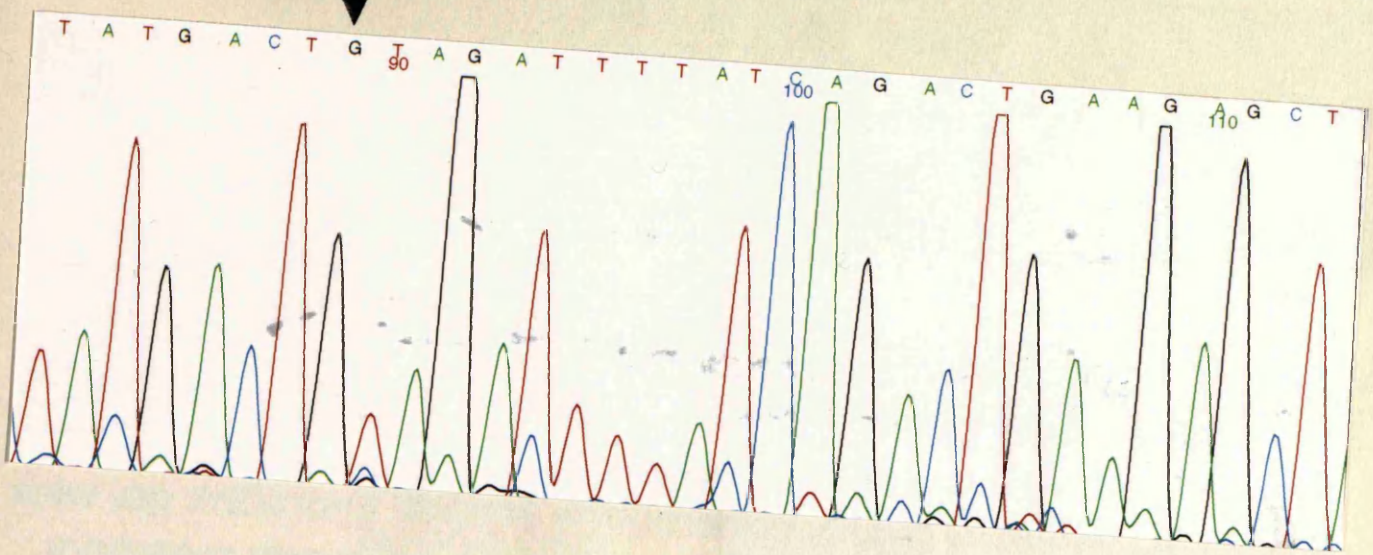
sense normal sequence : TTCCTATGACTGTAGATTTT

sense mutant sequence : TTCCTATGACTAGATTTT

Heterozygote : ********

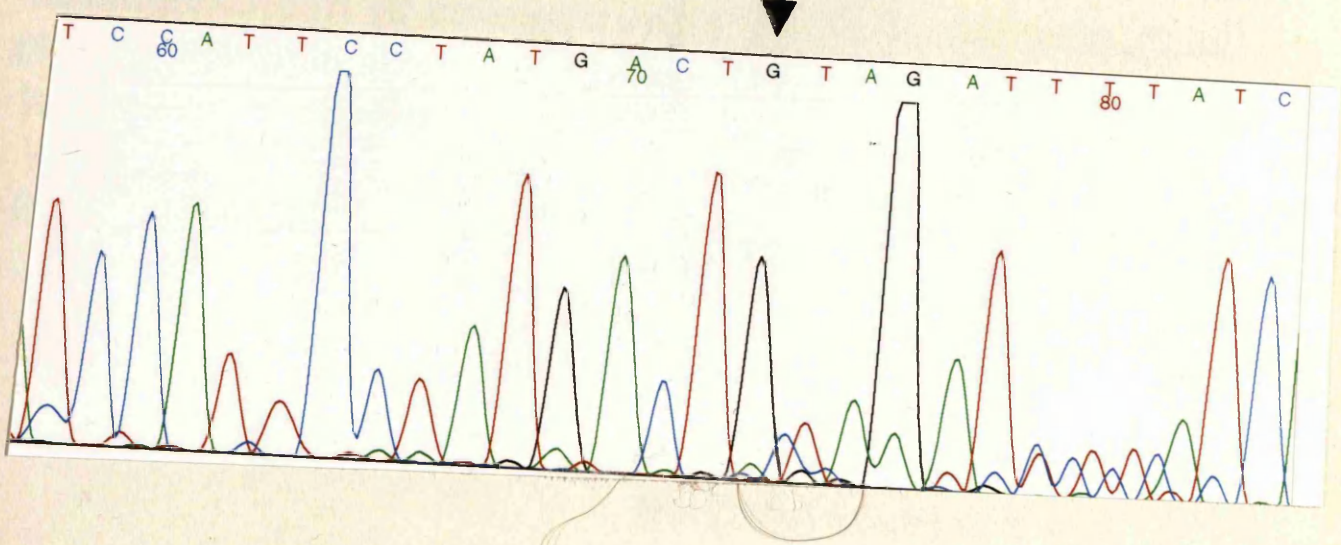
Figure 3.24.E

GT deletion



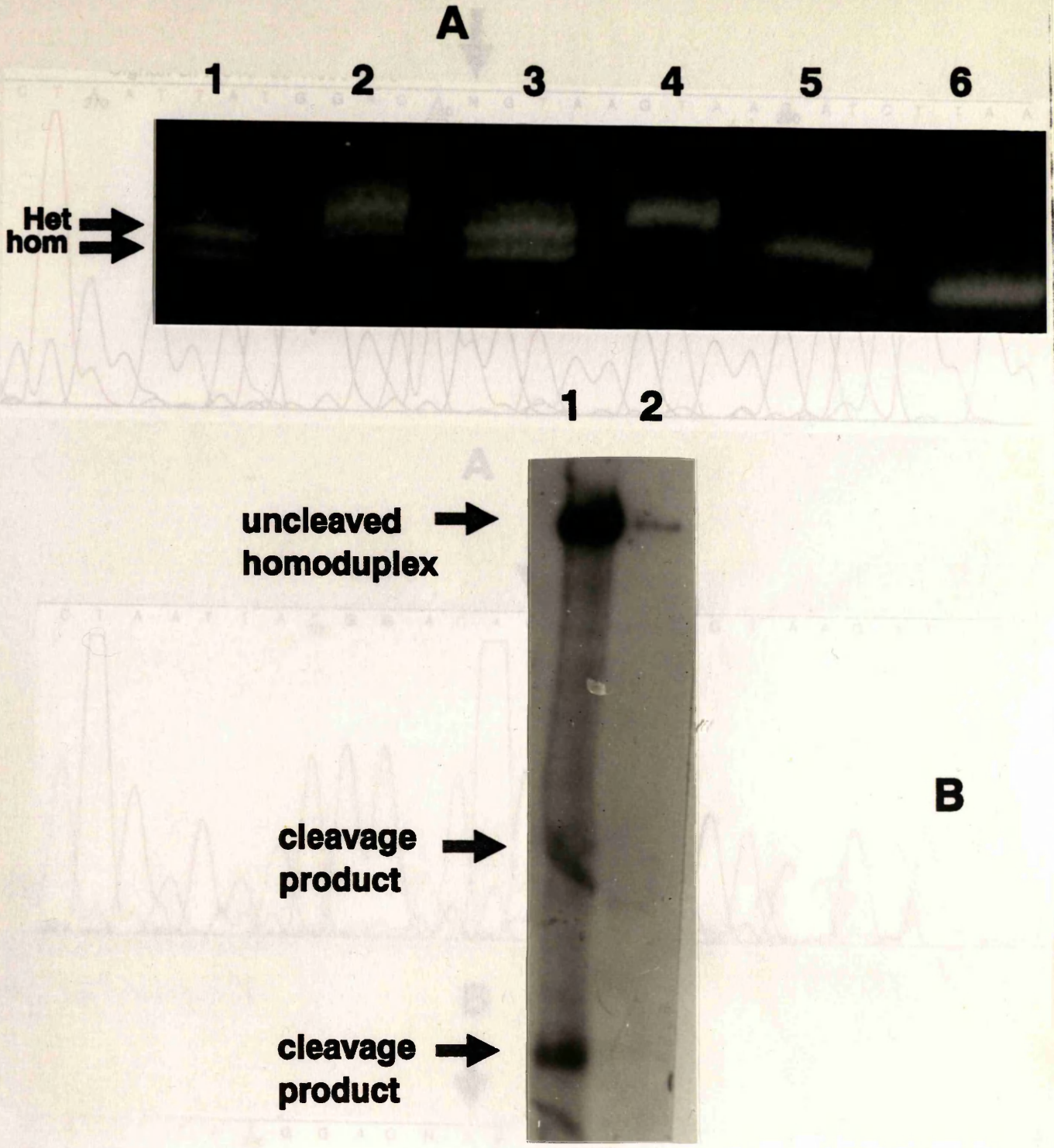
C

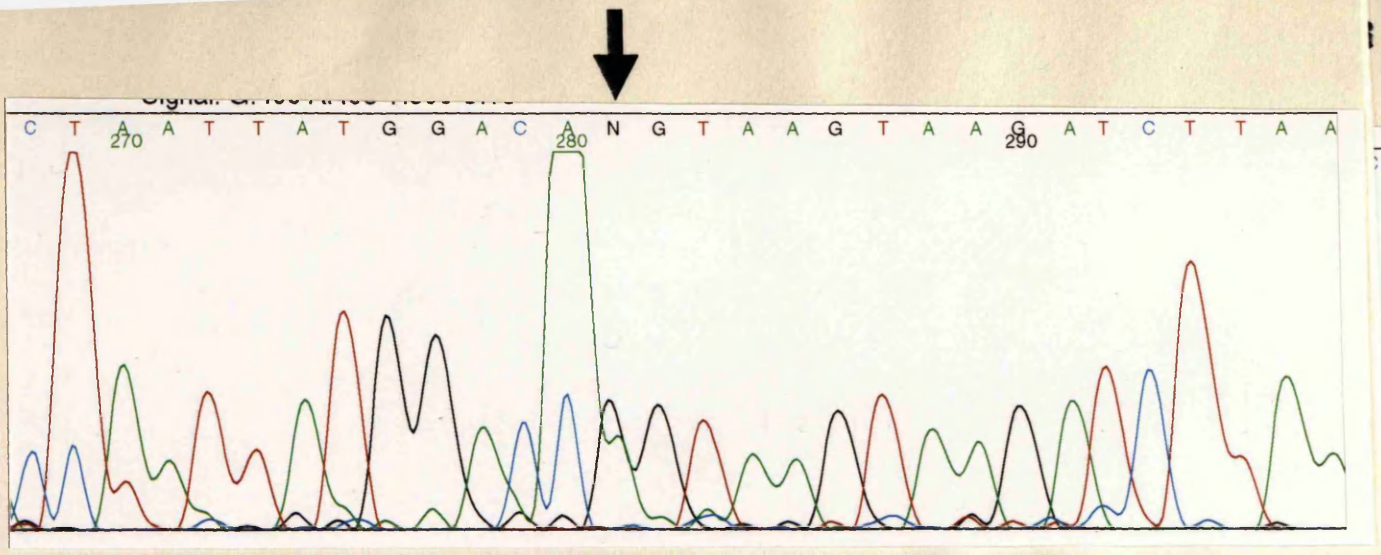
GT deletion



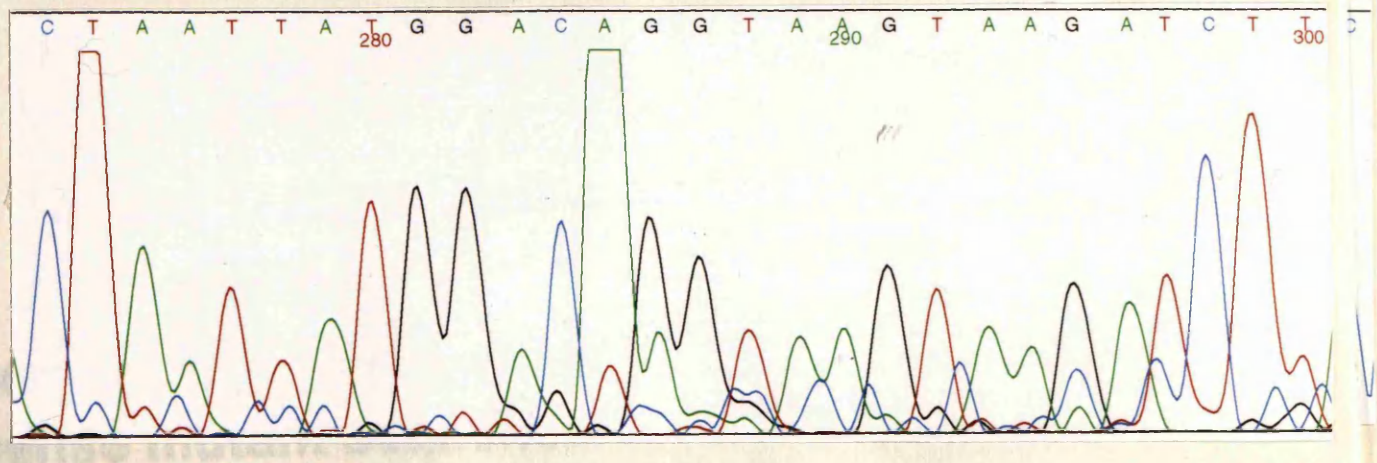
D

Figure 3.25 : Carrier detectionin exon 2 in the family Gla 7292. Figure A, shows an ethidium bromide stained hydrolink gel with results of heteroduplex detection analysis on family members. The lane numbers correspond to numbers beside the individuals in the pedigree shown in figure C and the hetroduplex and homoduplex bands are indicated by arrows. Figure B, shows the results of CCM analysis on the mother (lane 1) and one sister (3), (lane 2), after modification of the duplexes by hydroxylamine. The probes were internally labelled and the cleavage products in both lanes are indicated by arrows. Figure D, shows part of chromatograms from an automated sequencer , showing the sequence of the sense strand of the mother(1)(A), sister (3)(B) and index case (2)(C) from the family with a G/A substitution in exon 2. The position of the substitution is indicated by an arrow and normal and mutant sequences are shown and compared. No DNA was available from the second sister (4) for CCM or sequence analysis.

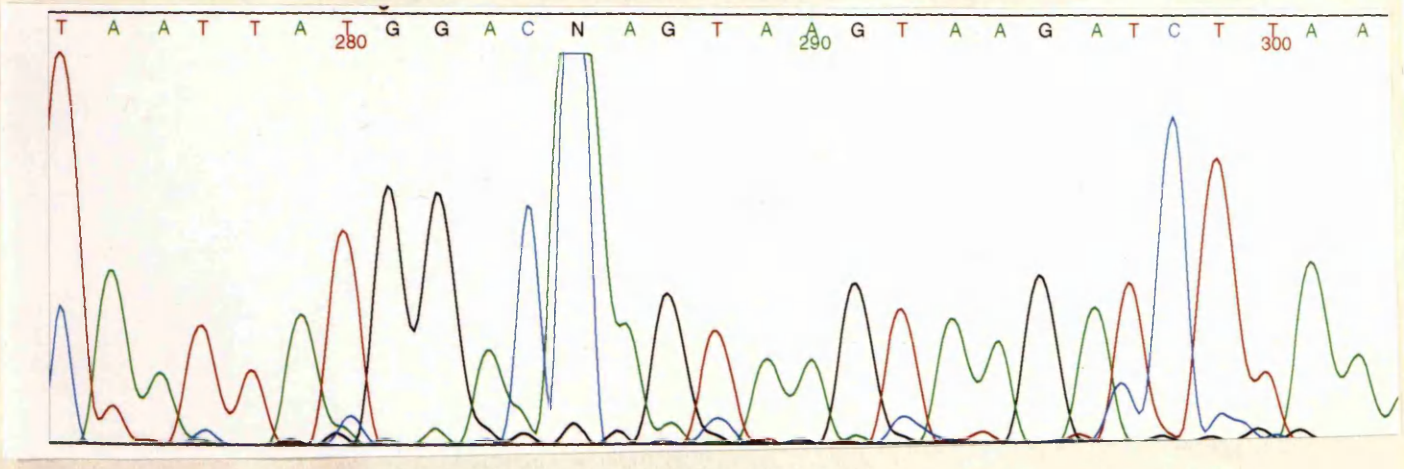

A
1
2
3
4
5
6
Het
hom
1
2
uncleaved
homoduplex
B
cleavage
product
cleavage
product
1
C
2
3
4



A



B

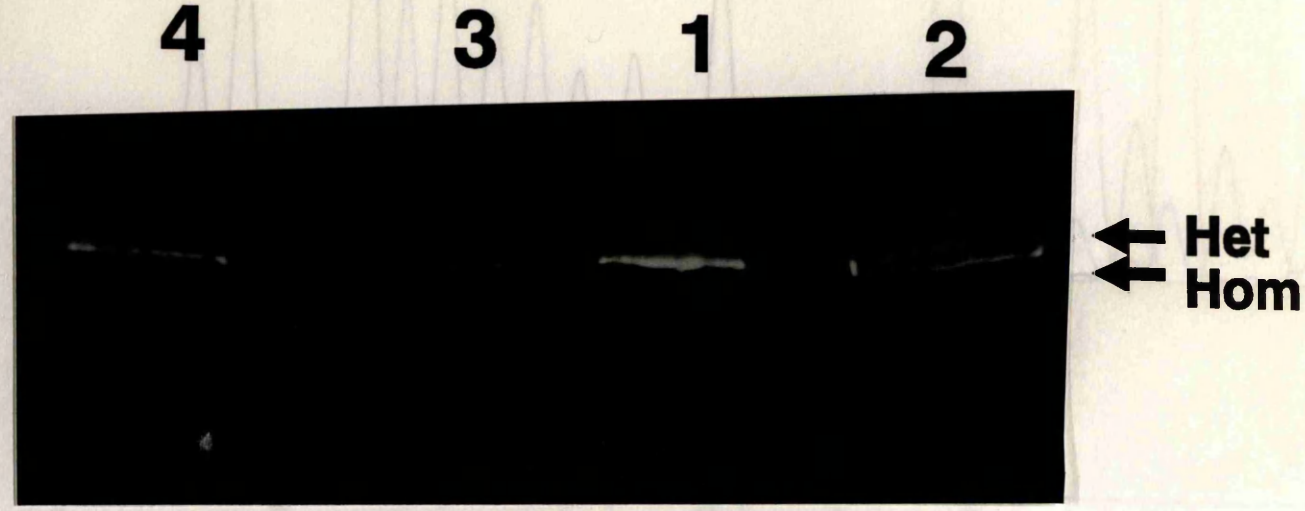


C

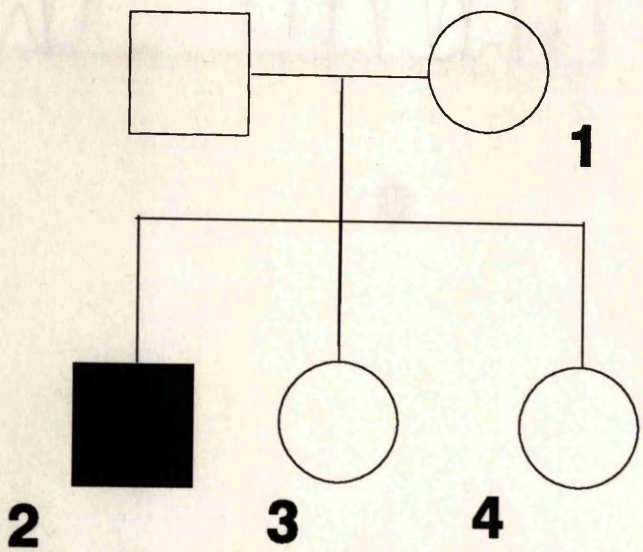
Figure 3.25.D

sense normal sequence: ATGGACAGGTAAGTAA
sense mutant sequence: ATGGACAAGTAAGTAA
heterozygote : A/G

Figure 3.26 : Carrier detection in exon 7 in the family HGPRT North Mymms. Figure A, shows an ethidium bromide stained hydrolink gel with the results of heteroduplex detection analysis on family members.The lane numbers correspond to numbers beside the individualsin the pedigree shown in figure B and the hetroduplex and homoduplex bands are indicated by arrows. The CCM analysis was uninformative in this family. Figure C shows part of a chromatogram showing sequence of the sense strand of exon 7 from the mother (1)(A), and sister (3)(B), of the index case. The position of the C-T substitution present in the index case is indicated by an arrow. DNA was not available from the index case or other family members for sequence analysis.

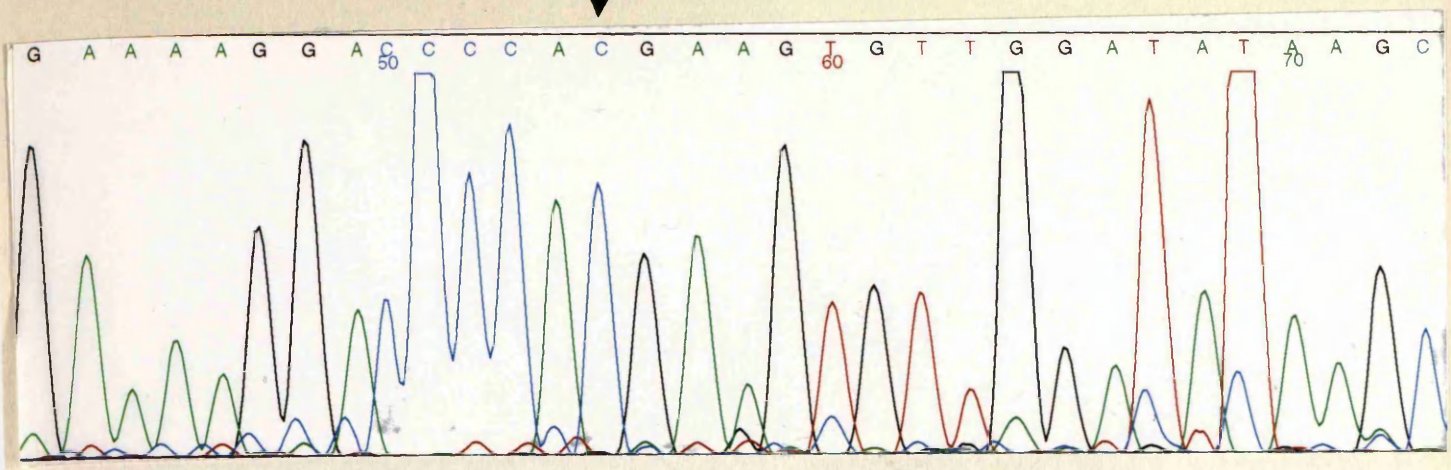


A

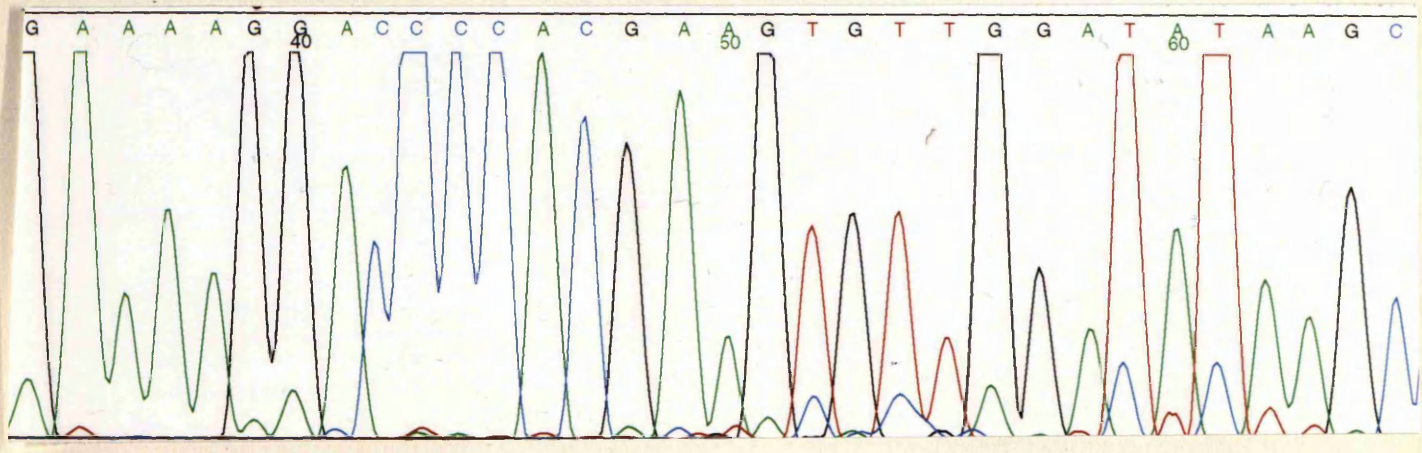


B

C /T mutation in HGPRT North Mymms



A



B

sense normal sequence : GGACCCCACGAAGTGTT
sense mutant sequence : GGACCCCATGAAGTGTT
Heterozygote : c/t

Figure 3.26.C

Figure 3.27 : Carrier detection in exon 5 in the family HGPRT 10195. Figure A, shows an ETBR stained hydrolink gel with the results of heteroduplex detection analysis on the maternal DNA. The hetroduplex bands are indicated by arrows. Figures B and C, shows a positive and negative image of the results of CCM analysis on the mother (lane 1) and a control sample (lane 2), after modification of the duplexes by adding hydroxylamine. The probes were end-labelled and the cleavage products are indicated by arrows. No DNA was available for the index case from this family and only maternal DNA was available for analysis at this point.

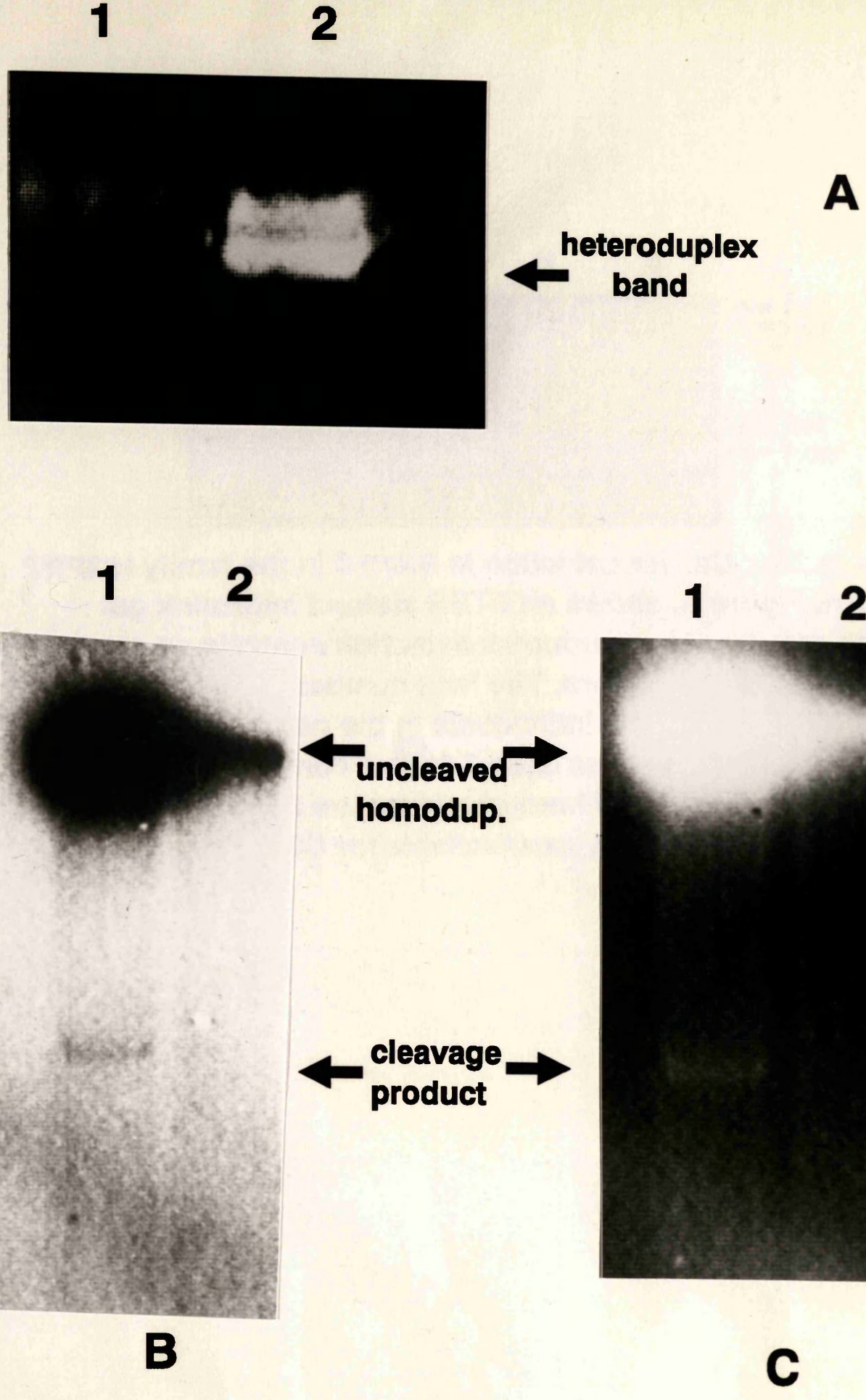
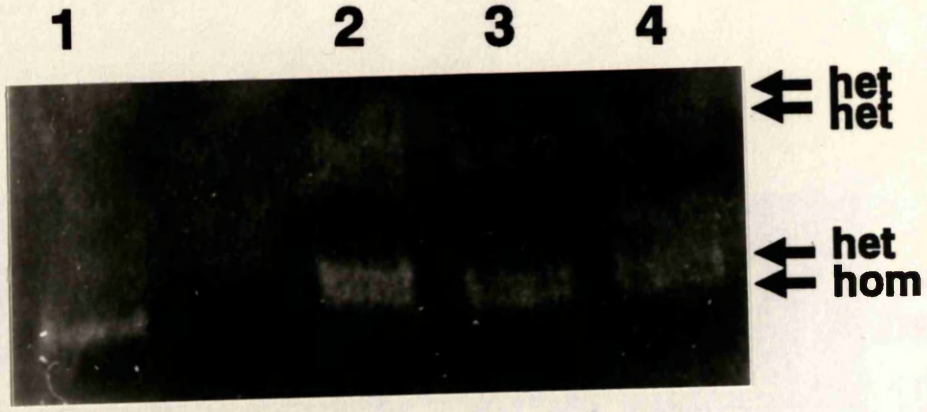

1
2
A
heteroduplex
band
1
2
uncleaved
homodup.
cleavage
product
B
1
2
C

Figure 3.28 : Carrier detection in exon 5 in the family HGPRT Runcorn. Figure A, shows an ETBR stained hydrolink gel with the results of heteroduplex detection analysis on exon 5 DNA from family members. The lane numbers correspond to the numbers beside the individuals in the pedigree shown in figure B and lane 1 shows exon 5 from a control individual. The heteroduplexes and homoduplexes are indicated by arrows. Insufficient DNA was available for CCM or sequence analysis in this family.

A



B

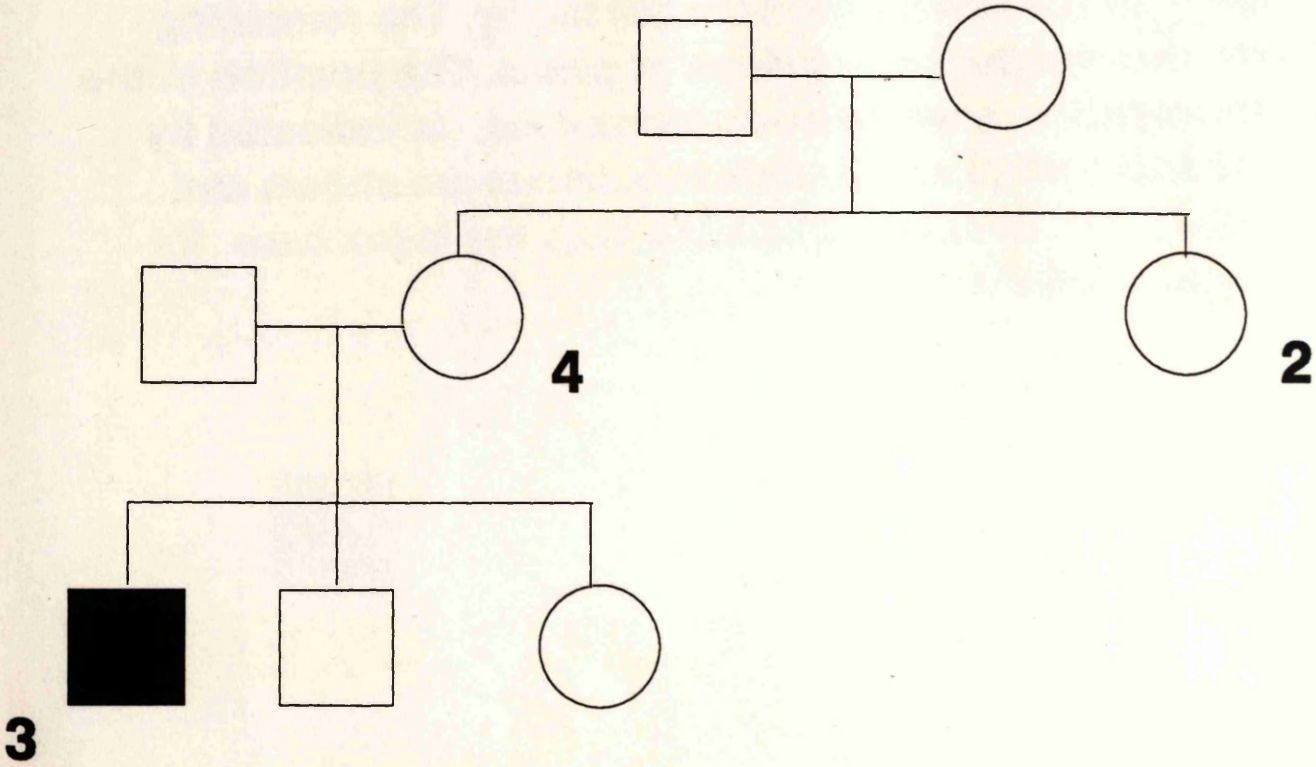
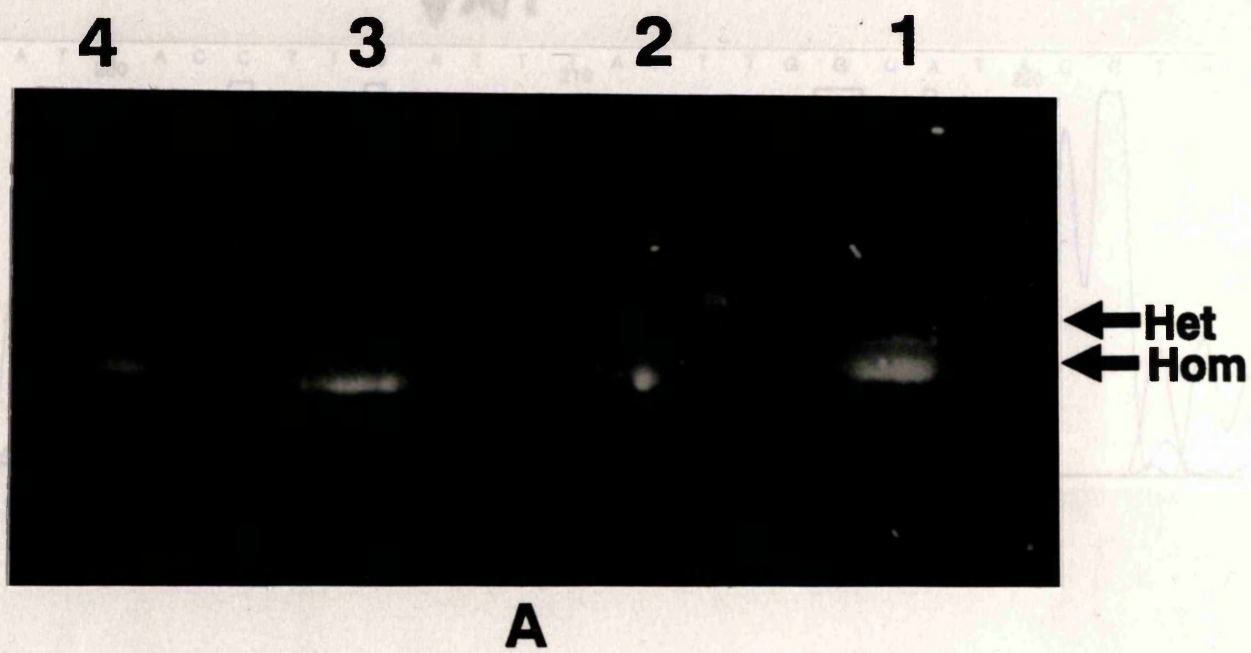
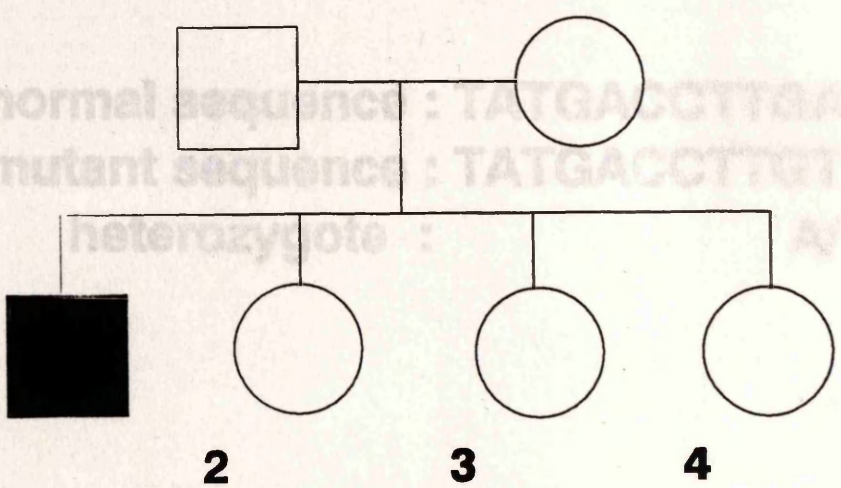


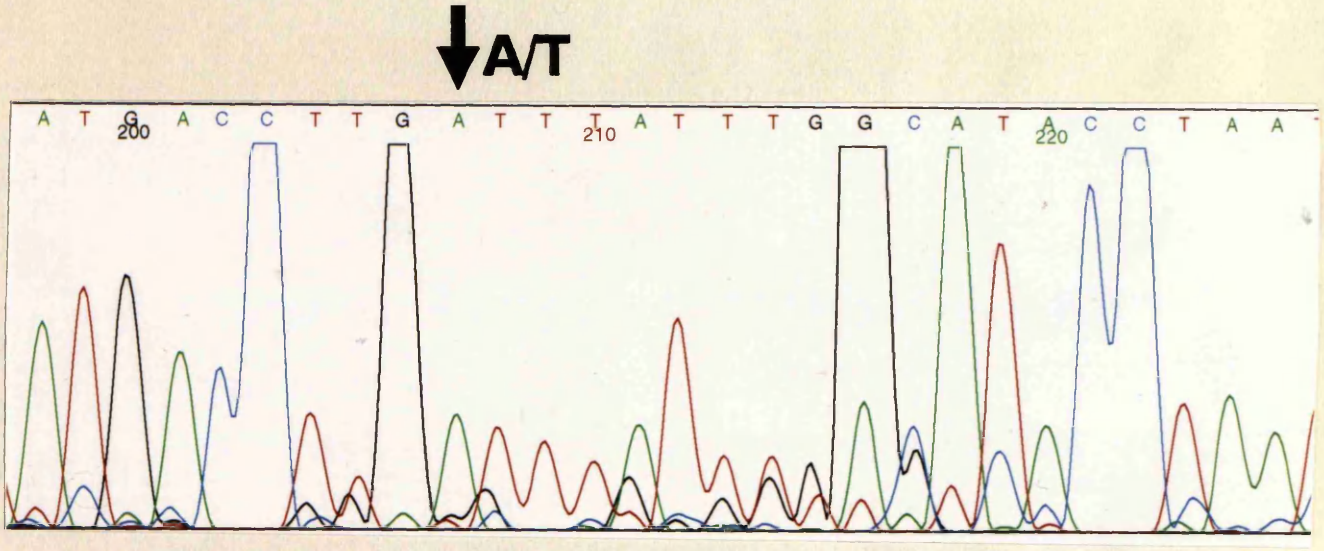
Figure 3.29 : Carrier detection in exon 2 in the family HGPRT Mashad. Figure A, shows an ETBR stained hydrolink gel with the results of heteroduplex detection analysis on family members. The lane numbers correspond to numbers beside the individuals in the pedigree shown in figure B and the hetroduplex and homoduplex bands are indicated by arrows. HDHG analysis was uninformative in this family. Figure C, shows part of a chromatogram from an automated sequencer , showing the sequence of the sense strand of exon 2 of one sister (3) of the index case from the family. The remaining sisters showed the same pattern of peaks. The position of the A/T substitution, present in the index case, is indicated by arrows and normal and mutant sequences are shown and compared. No DNA was aviailable from the index case for sequence analysis .



A



B



sense normal sequence : TATGACCTTGATTT
sense mutant sequence : TATGACCTTGTTTT
heterozygote : A/T

Figure 3.29.C

Figure 3.30 : Carrier detection in exon 4 in the family HGPRT Gla 7293. Figure B shows an ethidium bromide stained agarose gel with results of PCR analysis on family members. The lane numbers correspond to numbers beside the individuals in the pedigree shown in figure A and the mutant larger and normal bands are indicated. A 1kb ladder is shown in lane L.

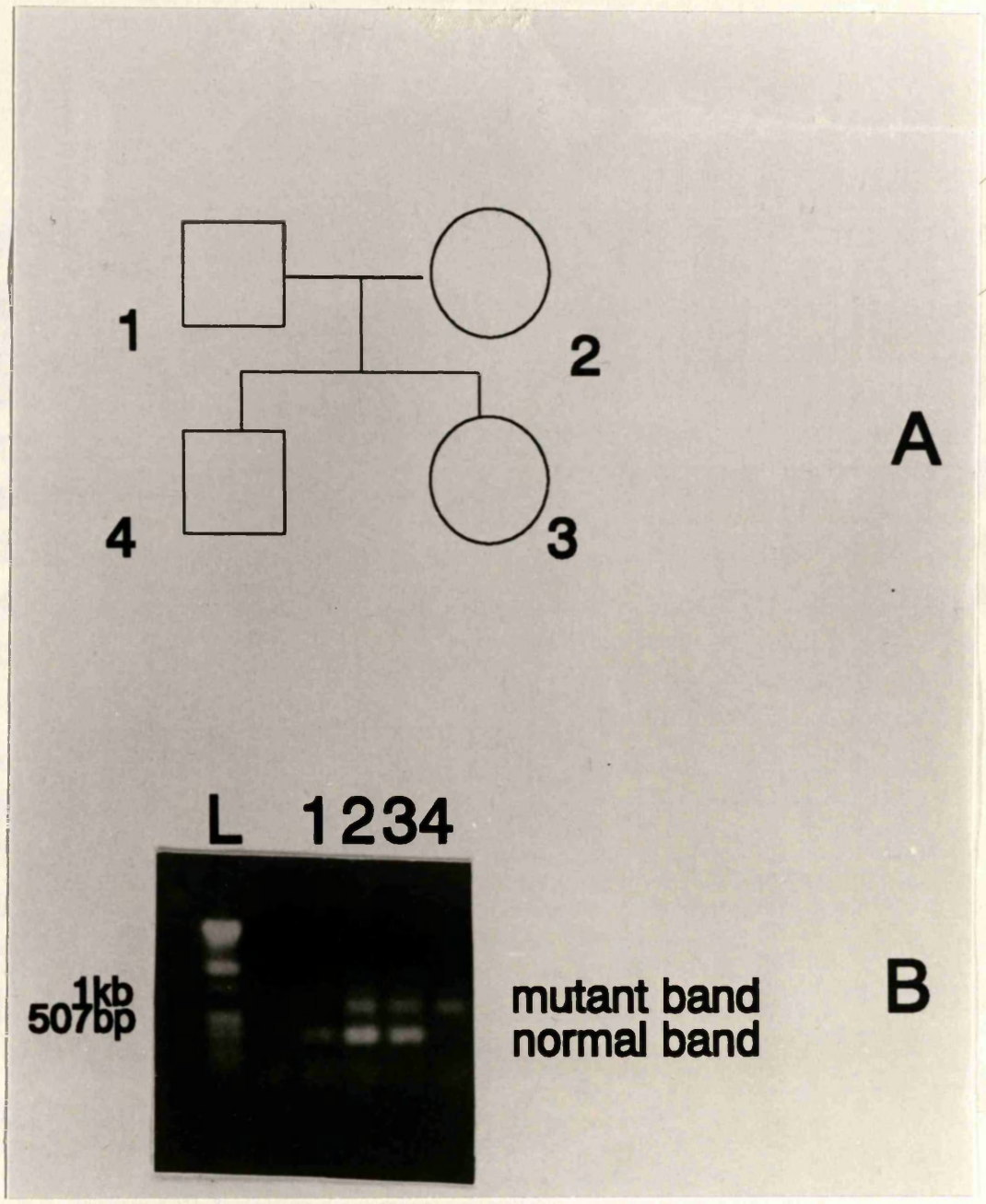
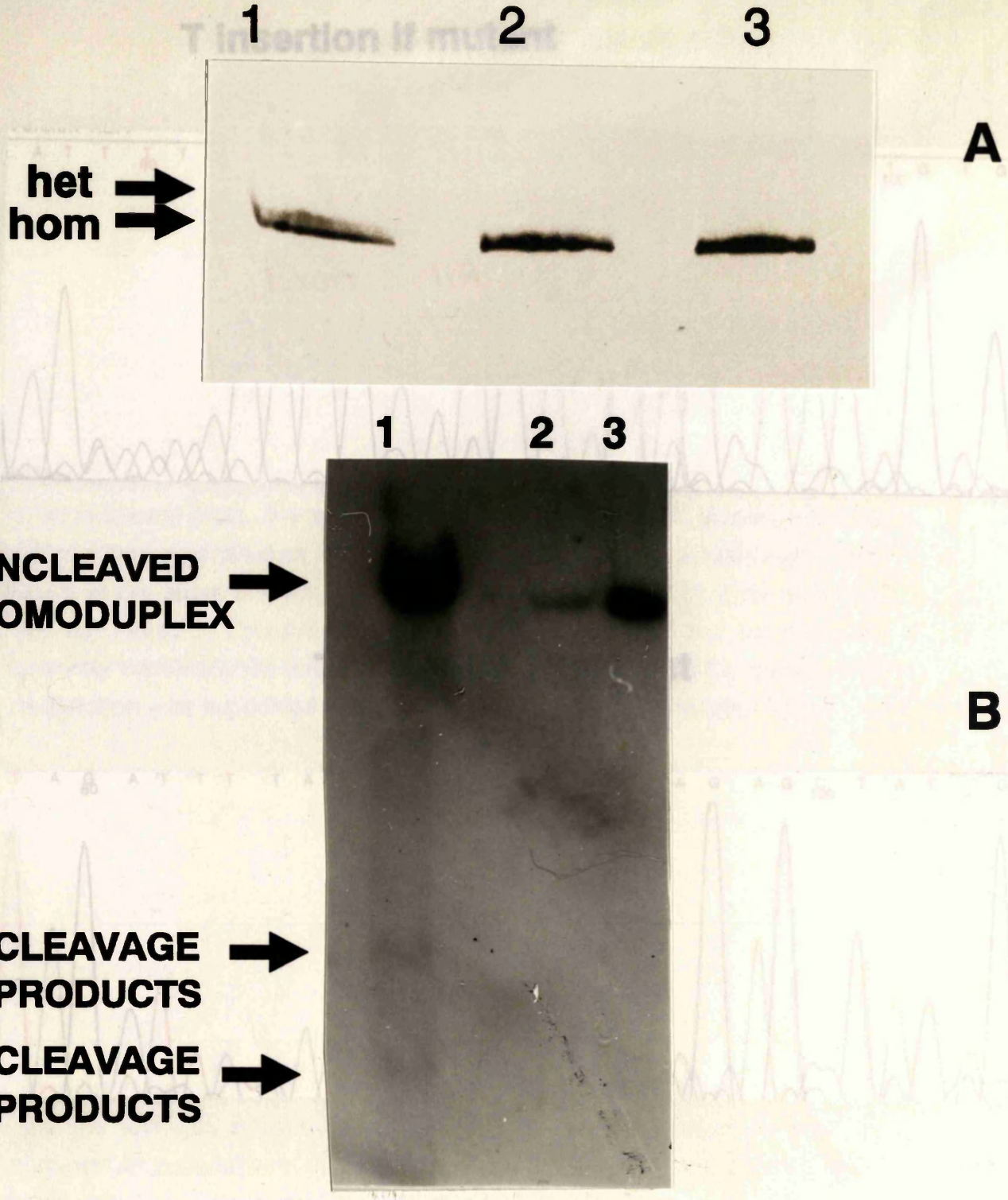
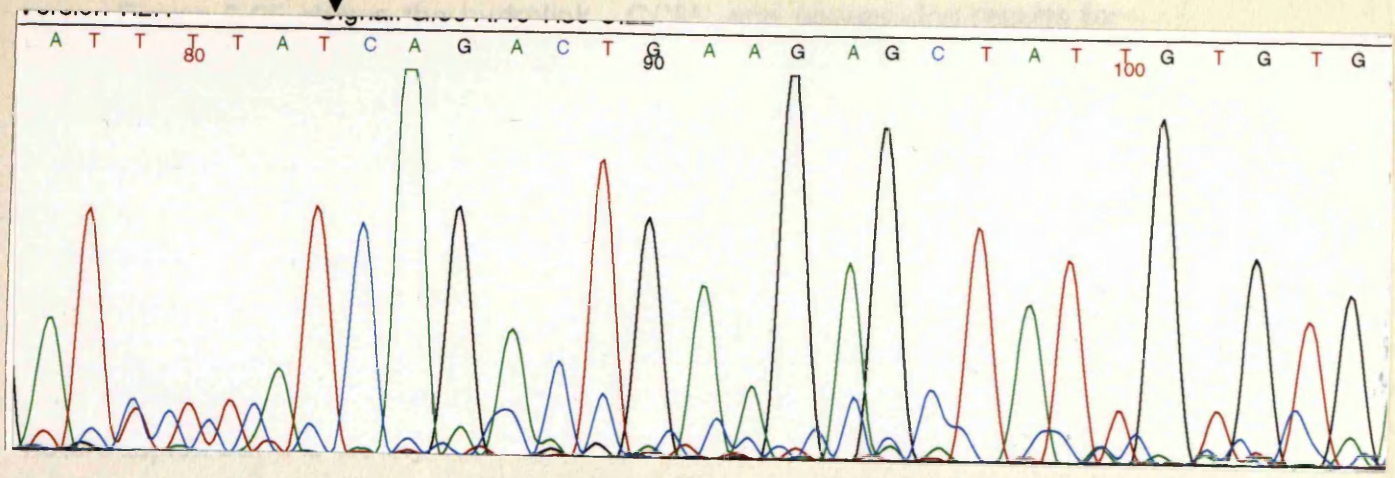

1
2
4
3
A
L 1234
1kb
507bp
mutant band
normal band
B

Figure 3.31 : Carrier detection in exon 3 in the family HGPRT Codicote. Figure A shows the -ve image of an ethidium bromide stained hydrolink gel with the results of heteroduplex detection analysis on family members.The lane numbers correspond to numbers beside the individuals in the pedigree shown in figure C and the heteroduplex and homoduplex bands are indicated by arrows. Figure B, shows the results of CCM analysis on the index case(1)(lane 1), sister (2)(lane 2), and aunt of the index case (3)(lane 3), after modification of the duplexes by addition of hydroxylamine. The probes were internally labelled and the cleavage products in lane 1 are indicated by arrows. No DNA was available from the other family members for HDHG or CCM analysis. Figure D, shows part of chromatograms from an automated sequencer , showing the sequence of the sense strand of exon 3 of the sister (2) and aunt (3) of the index case (chromatograms A and B respectively). No DNA was available from the index case or his mother for sequence analysis. The position of the potential T insertion is indicated by arrows and normal and mutant sequences are shown and compared.

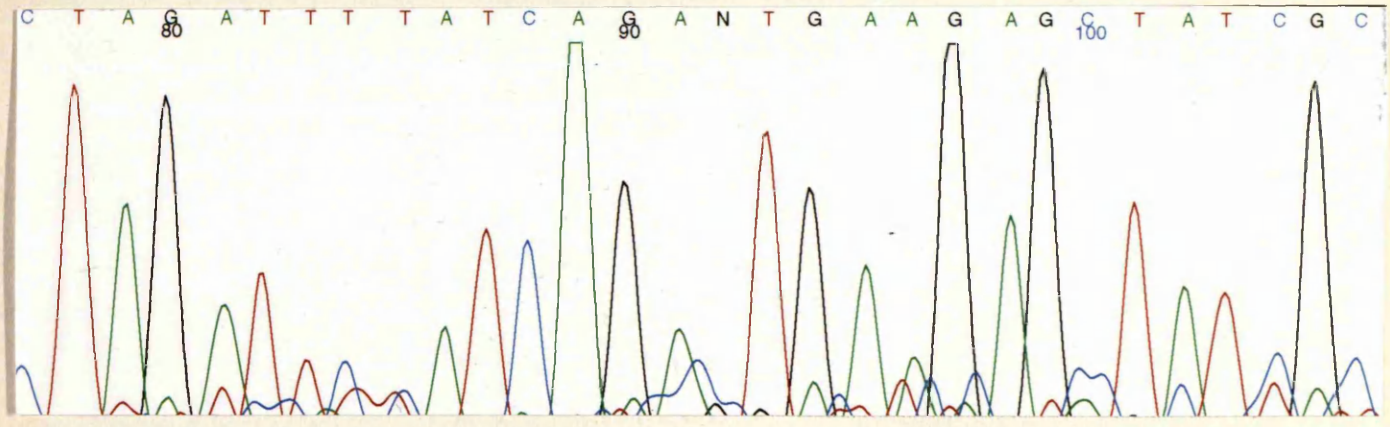

1
2
3
het
hom
A
1
2
3
UNCLEAVED
HOMODUPLEX
B
CLEAVAGE
PRODUCTS
CLEAVAGE
PRODUCTS
C
3
1
2

T insertion if mutant



A

T insertion if mutant



B

sense normal sequence : TTTTATCAGACTGAAGAG

sense mutant sequence : TTTTATTCAGACTGAAGAG

Heterozygote : ******************

Figure 3.31.D

Figure 3.25 shows the hydrolink , CCM and sequencing results for family members from the pedigree 7292. Using direct mutation detection strategies this individual was found to have a point mutation in exon 2 which could be seen as a heteroduplex band on hydrolink gels and as a cleavage product after modification with hydroxylamine. DNA was available for the mother(1) and one sister (3) but only a small quantity of frozen lyphoblastoid cells were available for the other sister (4), providing enough DNA only for hydrolink analysis. The hydrolink (figure A) and CCM results (figure B) are shown. The linkage analysis was uninformative for this family. A heteroduplex band is clearly seen for the mother (1) and sister (3) while this band is absent from the second sister (4). The chemical cleavage results show a cleavage product for the mother (1) and sister (3) although these bands in the sister are very faint. (Lanes 1 and 2 , figure B) while sister (4) was not tested . This indicated that the mother(1) and one sister (3) are definitely carriers while the second sister (4) is probably not a carrier. This designation was supported by the automated sequencing results (E).

Figure 3.26 shows the hydrolink (figure A) and sequence results(C) from the mother and sisters of the index case with the mutation HGPRT North Mymms(pedigree 10188). Linkage analysis was informative and is shown in figure 3.23.d. The linkage analysis suggests that one sister(3) (4 in the linkage pedigree) carries the affected maternal chromosome(allele A), while the other sister does not. CCM analysis was uninformative for this exon. The hydrolink and sequence data show that the mother and sisters do not carry the mutation. Figure C, shows the sequence of this part of the exon 7 from the mother(1)(A) and sister (3)(B)of the index case (2). DNA was not available to show the mutation in the index case by sequence analysis but coupled with the hydrolink and linkage data, it appears that the index case is a new mutation.

Figure 3.27 shows the and hydrolink results (figure A) and CCM analysis (B) and (C) from the mother of the index case from the pedigree 10195. Very little DNA was available from the index case and only PCR of the individual exons from this boy was possible to exclude large length mutations. The direct mutation detection was carried out on the maternal DNA and a mutation was indicated in exon 5 (figure A) with the presence of a heteroduplex band in exon 5 . The CCM results (B) and (C) also shows a cleavage product in this exon. The mother is shown by direct mutation

detection probably to be a carrier. Automated sequencing showed no definate mutation in the mother and no further DNA was available for hydrolink analysis on the other family members and the linkage analysis was uninformative, so no further data could be given to this family.

Figure 3.28 Shows a partial pedigree (figure B) with the hydrolink data (figure A) for several family members with the mutation HGPRT Runcorn (pedigree 10199). Small quantities of DNA were available for the index case and insufficient DNA was left to carry out linkage analysis or CCM. The HDHG results are shown for exon 5 of the index case (3) mother (4) and aunt (2). All three individuals show the presence of a heteroduplex band indicated by an arrow. The carrier status of these family members could however not be confirmed as sequence could not be obtained for this exon as insufficient DNA was available for sequence analysis, but the hydrolink results are among the clearest found.

Figure 3.29 shows the HDHG (figure A), partial pedigree (figure B) and sequence data (figure C) for individuals from the family with the mutation HGPRT Mashad (pedigree 10197). The linkage data was uninformative due to the absence of maternal DNA and the CCM protocol did not yield a cleavage product for any individuals after modification with either chemical. The hydrolink data indicated that none of the sisters were carriers with all the sisters (lanes 2,3 and 4) appearing to show a single band. No DNA remained for sequencing of the index case DNA but the sequence of the sisters DNA confirmed the absence of the mutation as no double peaks were seen at the point of the mutation as were seen in the other carrier females from other families. The sequence from one sister only is shown, but all sisters yielded similar chromatograms.

Figure 3.30 shows the PCR amplification products from exon 4 from the family with pedigree 7293. Due to the presence of a large insertion in exon 4 the carrier status of female family members could be easily assigned by the presence of the large band in addition to a normal sized exon 4 band. These results confirm the linkage analysis.

Figure 3.31 Shows the HDHG (figure A), CCM (figure B) and sequence (D) results from the family with the mutation HGPRT Codicote (pedigree 10198). A partial pedigree is shown in figure C. The linkage

analysis was uninformative due to homozygosity of the marker(figure 3.23.g). No DNA was available for further analysis of the maternal DNA, but as a second male fetus was terminated and was found to have Lesch-Nyhan syndrome, it can be concluded that the mother is an obligate carrier. Exon 3 was problematic for CCM but cleavage products were seen in the index case but not in the sister (2) or aunt(3). The HDHG shows the presence of a double band in the index case DNA but not in the DNA from the aunt(3) or sister(2). The sister and aunt of the index case were sequenced as no further DNA was available from the mother or index case. No mutation was seen in either and as a T insertion would result in double peaks thereafter in the sequence it can be presumed that no mutation is present in these samples. DNA was not available for the remainder of the female family members.

3.2 Duchenne Muscular Dystrophy.

3.2.1 Screening for Point Mutations in DMD/BMD Patients With No Large Deletions.

Fourteen DMD/BMD patients with no apparent deletions were studied using heteroduplex detection using HDHG and CCM analysis to screen 21 regions of the gene for the presence of small mutations. Ten of the boys had exons a-i previously screened using SSCP analysis but no mutations had been found (Kataki, 1992). The remaining boys had been screened only for the presence of large length mutations .

3.2.2 Heteroduplex Detection Using Hydrolink Gel Electrophoresis in the *DMD* Gene.

The 21 regions of the *DMD* gene were amplified in each boy by the modified PCR protocol described in 2.6., with control DNA added to each reaction tube to enable creation of a potential heterozygote state. After amplification, the duplexes were run on a hydrolink gel, the DNA stained with ethidium bromide and each exon examined for the presence of extra bands indicating the presence of heteroduplexes.

Three patterns were observed . The exons were scored either as definite negatives indicated by -; definite positives indicated by +; or suspicious indicated by ?. A suspicious score was recorded if the band was

abnormal in some way but did not have the presence of definite extra bands. This could be due to a thickening of the band relative to the others on the gel, if an extra band was seen present some distance from the homoduplex band or if the same exon from different boys showed a duplex band on the same gel indicating that a loading artefact may have occured resulting from incorrect formation of the wells on the gel. Each exon was analysed at least twice using two separate amplification products with two different control DNA's run on separate gels.

The results from these gels are summarised in Tables 3.5.a,and 3.5.b. The positive results are shown in figure 3.32.a-c.

3.2.3 CCM Analysis of Suspicious *DMD* Exons.

CCM analysis using both hydroxylamine and osmium tetroxide was carried out on all exons which gave a suspicious or positive result with HDHG. The results obtained are summarised inTable 3.6.a and 3.6.b.

Of the exons analysed in the first PCR reaction amplifying exons a-i two revealed a cleavage and several gave a possible cleavage result that was difficult to interpret. The positive cleavage results are shown in figure 3.33 A and B. In the second set of PCR reactions no definite cleavages and 4 suspicious results were seen.

Figure 3.33.a shows the cleavage of exon a after modification with osmium tetroxide from the index case of pedigree 6397. Figure 3.33.b shows cleavage after modification with hydroxylamine of exon b of the index case from the pedigree 5117. Unclear cleavage results were obtained from; exon a of the index case from the pedigree 5113, exon c of the index case from the pedigree 5104 and exon f of the index case from the pedigree 5117 , exon i of the index case from the pedigree 5108 and exon 60 of the index case from the pedigree 5100. Exon g could not be analysed using CCM due to the unavailability of primers. Three further potential cleavage events were seen, in exons 42 and Pm (after modification by osmium tetroxide and hydroxylamine respectively) , from the pedigree 4252 and exon 13 of the index case from the pedigree 5113, after modification by hydroxylamine.

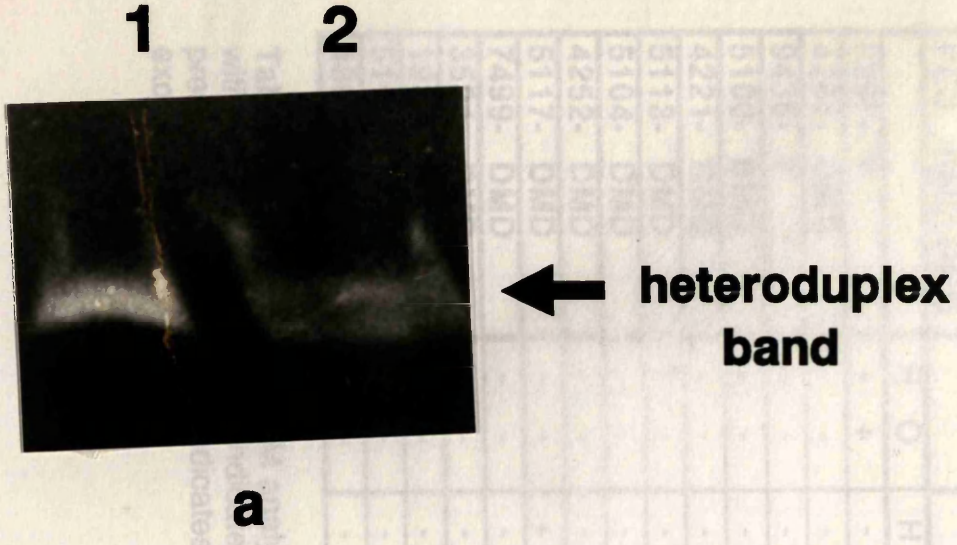
PED /BMD:DMD	A	B	C	D	E	F	G	H	I
6397- ?	+ +	? -	- -	- -	- -	- -	- -	- -	- -
4287- DMD	? -	- -	- -	- -	- -	- -	- -	- -	- -
9436- ?	? -	- -	- -	- -	- -	? -	- -	- -	- -
5100- BMD	- -	- -	- -	- -	- ?	- -	- -	- -	? -
4221- DMD	- -	- -	- -	- -	- -	- -	- -	- -	- -
5113- DMD	? ?	? -	- -	- -	- -	- -	- -	? -	- -
5104- DMD	- -	- -	? ?	- -	? -	- -	- -	- -	- -
4252- DMD	- -	- -	- -	- -	- -	- -	- -	- -	- -
5117- DMD	- -	+ +	- -	- -	- -	? ?	- -	- -	- -
7499- DMD	- -	- -	- -	- -	? -	- -	- -	- -	- -
3591- DMD	- -	? -	- -	- -	- -	- -	- -	- -	- -
1987- BMD	- -	? -	- -	- -	- -	- -	- -	- -	- -
5108- DMD	? -	- -	- -	- -	- -	- -	- -	- -	? ?
4861- DMD	? -	- -	- -	? -	- -	- -	? -	- -	- -

Table 3.5.a. Results of HDHG analysis of exons a-i of 14 boys with DMD/BMD. (-) indicated the absence of a heteroduplex band, (+), indicates the presence of a heteroduplex band, and (?), indicates an uncertain result with the presence of a band with an abnormal appearance.

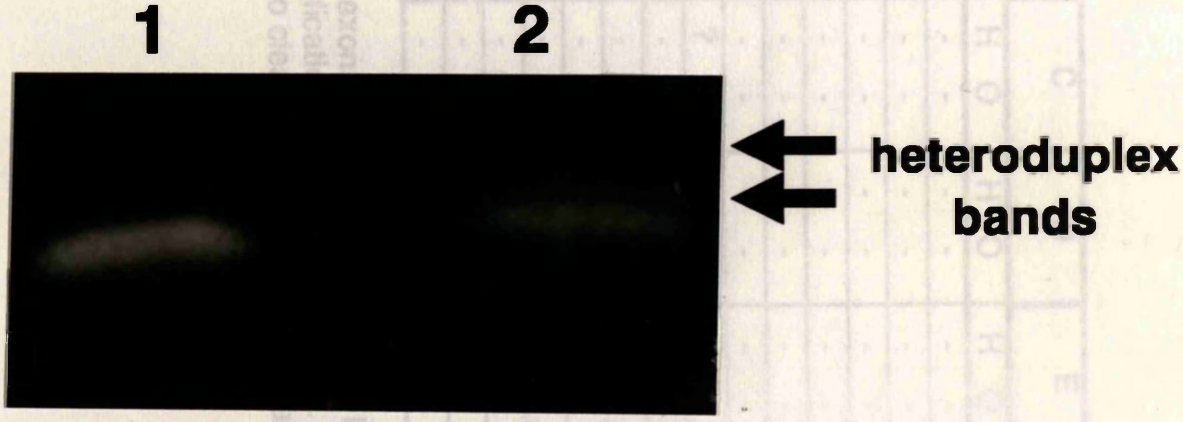
PED /BMD:DMD	PM	3	6	13	42	43	44	47	52	53	60
6397- ?	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
4287- DMD	- -	- -	- -	- -	- -	- ?	- -	- -	- -	- -	- -
9436- ?	- -	- -	- -	- -	- -	- -	? -	- -	- -	- -	- -
5100- BMD	- -	- -	- -	- -	- -	- -	? ?	- -	- -	- -	? ?
4221- DMD	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
5113- DMD	- -	- -	- -	+ +	- -	- -	- -	- -	- -	- -	- -
5104- DMD	? -	- -	- -	- -	- -	- -	- -	? -	- -	? -	- -
4252- DMD	- ?	- -	- -	- -	- ?	- -	- -	? ?	- -	- -	- -
5117- DMD	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
7499- DMD	- -	- ?	- -	- -	- ?	- -	- -	- -	- ?	- -	- -
3591- DMD	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
1987- BMD	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
5108- DMD	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -	- -
4861- DMD	- -	- -	- -	- -	- -	- ?	- -	- -	- -	- -	- -

Table 3.5.b. Results of HDHG analysis of exons from the second set of PCR reactions of 14 boys with DMD/BMD. (-) indicated the absence of a heteroduplex band, (+), indicates the presence of a heteroduplex band, and (?), indicates an uncertain result with the presence of a band with an abnormal appearance. Exon 50 was not tested as results from analysis in this exon in all individuals were unclear as all results appeared positive.

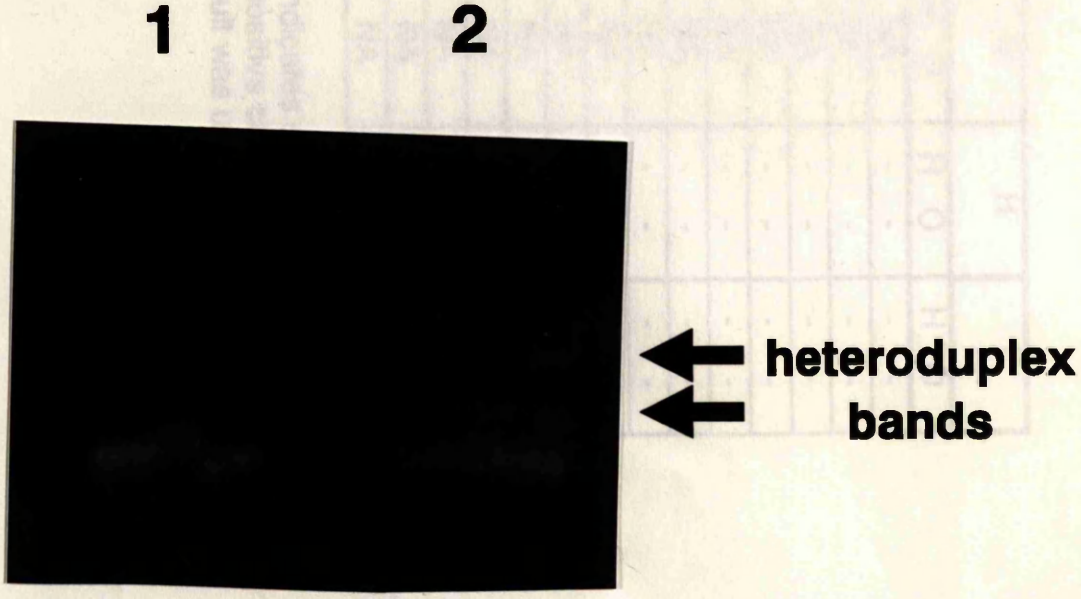
Figure 3.32 : Ethidium bromide stained hydrolink gels showing exons from index cases with DMD, which gave heteroduplexes. The figures show the formation of heteroduplex bands in the index cases in lanes 2, against normal control DNA, in lanes 1. (a) shows exon a of the index case from pedigree 6397, (b) shows exon b of the index case from pedigree 5117 and (c) shows exon 13 of the index case form pedigree 5113. The heteroduplex bands are indicated by arrows.


1
2
heteroduplex band

a


1
2
heteroduplex bands

b


1
2
heteroduplex bands

c

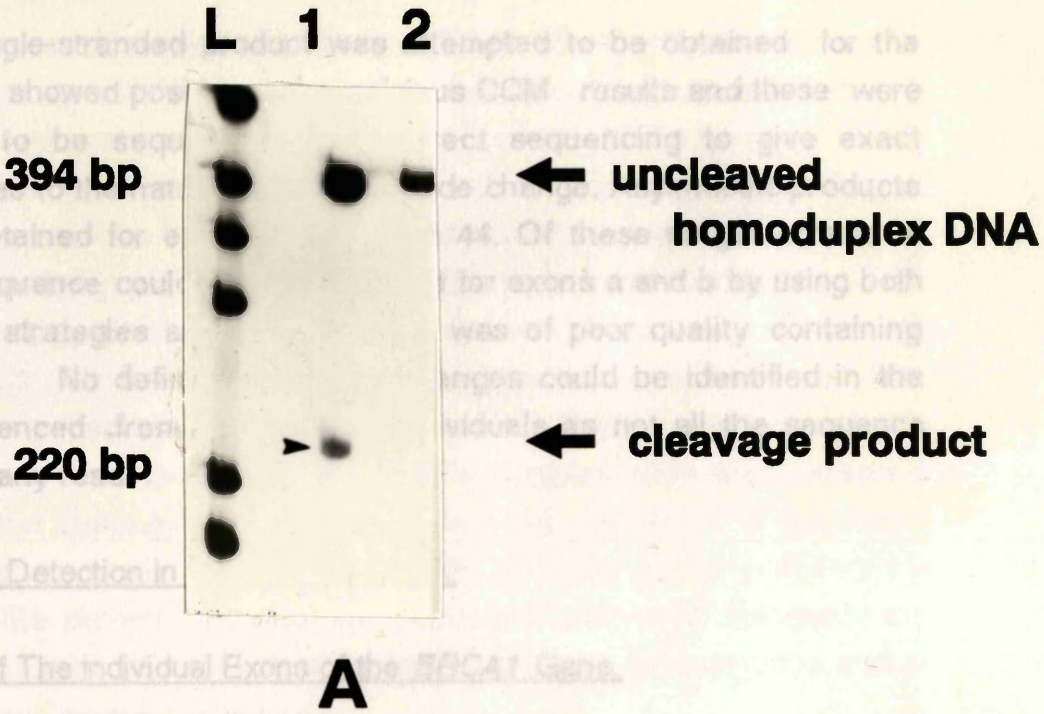
PED /BMD:DMD	A		B		C		D		E		F		G		H		I	
	H	O	H	O	H	O	H	O	H	O	H	O	H	O	H	O	H	O
6397- ?	-	+	-	-	-	-	-	-	-	-	-	-	NA		-	-	-	-
4287- DMD	-	-	-	-	-	-	-	-	-	-	-	-	NA		-	-	-	-
9436- ?	-	-	-	-	-	-	-	-	-	-	-	-	NA		-	-	-	-
5100- BMD	-	-	-	-	-	-	-	-	-	-	-	-	NA		-	-	-	-
4221- DMD	-	-	-	-	-	-	-	-	-	-	-	-	NA		-	-	-	-
5113- DMD	?	-	-	-	-	-	-	-	-	-	-	-	NA		-	-	-	-
5104- DMD	-	-	-	-	?	-	-	-	-	-	-	-	NA		-	-	-	-
4252- DMD	-	-	-	-	-	-	-	-	-	-	-	-	NA		-	-	-	-
5117- DMD	-	-	+	-	-	-	-	-	-	-	-	?	NA		-	-	-	-
7499- DMD	-	-	-	-	-	-	-	-	-	-	-	-	NA		-	-	-	-
3591- DMD	-	-	-	-	-	-	-	-	-	-	-	-	NA		-	-	-	-
1987- BMD	-	-	-	-	-	-	-	-	-	-	-	-	NA		-	-	-	-
5108- DMD	-	-	-	-	-	-	-	-	-	-	-	-	NA		-	-	?	-
4861- DMD	-	-	-	-	-	-	-	-	-	-	-	-	NA		-	-	-	-

Table 3.6.a. Results of CCM analysis of exons a-i from 14 boys with DMD/BMD. H indicates modification PCR product with hydroxylamine and O indicates modification by osmium tetroxide. + indicates a positive cleavage indicating the presence of a mismatch, -indicates that no cleavage occured. ?, indicates that the result was unclear. NA indicates that the exon was not tested.

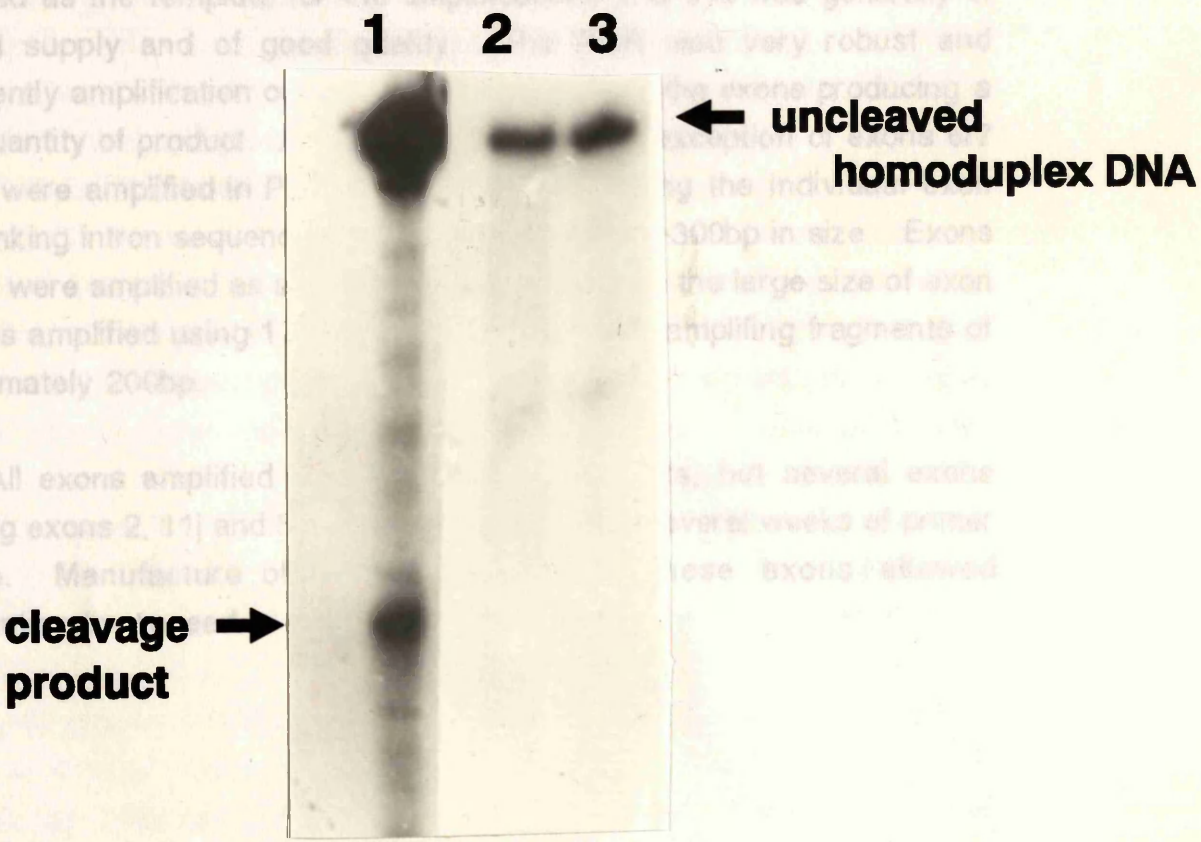
PED /BMD:DMD	PM		3		6		13		42		43		44		47		50		52		53		60	
	H	O	H	O	H	O	H	O	H	O	H	O	H	O	H	O	H	O	H	O	H	O	H	O
6397- ?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4287- DMD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9436- ?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5100- BMD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	?
4221- DMD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5113- DMD	-	-	-	-	-	-	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5104- DMD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4252- DMD	?	-	-	-	-	-	-	-	-	?	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5117- DMD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7499- DMD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3591- DMD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1987- BMD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5108- DMD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4861- DMD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3.6.b. Results of CCM analysis of exons from the second PCR reactions(PM-60), from 14 boys with DMD/BMD. H, indicates modification PCR product with hydroxylamine and O indicates modification by osmium tetroxide. +, indicates a positive cleavage indicating the presence of a mismatch, -,indicates that no cleavage occured. ?, indicates that the result was unclear.

Figure 3.33 : Autoradiograph showing cleavage of end-labelled probes in exons of patients with DMD/BMD. Figure 3.33.a shows cleavage after modification by osmium tetroxide of exon a of the index case from the pedigree 6397, the cleavage product is marked by an arrow. Figure 3.33.b shows cleavage after modification by hydroxylamine of exon b of the index case from pedigree 5117, the cleavage product is indicated by an arrow. The control samples are shown in lane 2 and in lanes 2 and 3 respectively.


L 1 2
394 bp
220 bp
uncleaved
homoduplex DNA
cleavage product

A


1 2 3
uncleaved
homoduplex DNA
cleavage
product

B

3.2.4 Sequencing of Suspicious *DMD* Exons.

A single-stranded product was attempted to be obtained for the exons which showed positive and suspicious CCM results and these were attempted to be sequenced using direct sequencing to give exact information as to the nature of the nucleotide change. Asymmetric products could be obtained for exons a, b, i, and 44. Of these single-stranded products sequence could only be obtained for exons a and b by using both sequencing strategies and the sequence was of poor quality containing many stops. No definite sequence changes could be identified in the exons sequenced from the affected individuals as not all the sequence could be clearly read.

3.3 Mutation Detection in the *BRCA1* Gene.

3.3.1 PCR of The individual Exons of the *BRCA1* Gene.

The individual exons of the *BRCA1* gene were amplified according to the protocol described in 2.7 using a touchdown PCR recommended by Miriad Genetics, the designers of the majority of the primers. Genomic DNA was used as the template for the amplifications and this was generally in plentiful supply and of good quality. The PCR was very robust and consistently amplification occured for the majority of the exons producing a large quantity of product. All of the exons with the exception of exons 6/7 and 11 were amplified in PCR products encompassing the individual exon plus flanking intron sequences giving products of 200-300bp in size. Exons 6 and 7 were amplified as a single product and due to the large size of exon 11 it was amplified using 17 overlapping primer sets amplifing fragments of approximately 200bp.

All exons amplified with the fresh primer sets, but several exons including exons 2, 11j and 5 ceased to amplify after several weeks of primer storage. Manufacture of new primer sets for these exons allowed amplification to proceed.

3.3.2 Screening for Molecular Pathologies in the *BRCA1* Gene.

Due to the large number of exons in the *BRCA1* gene, the number of DNA samples and to the small size of the amplification products produced by use of the chosen primers, it was decided to screen the gene by SSCP analysis. Any suspect exons from the patients was then sequenced using an ABI automated sequencer. Two SSCP gel conditions were used to obtain maximum screening efficiency , one gel was run at room temperature with 5% glycerol added to the gel and one gel was run in the cold room with no glycerol. A strategy was devised where the previously reported common mutations were screened for primarily in the family samples and the sporadic breast and ovarian tumours DNA samples were then screened using ASO dot blotting for these mutations. The remainder of the exons were then screened with exon 11 being firstly analysed as it encodes for the majority of the protein. Due to the autosomal nature of the gene any mutations found in the blood samples are likely to be heterozygous and in the carriers two sequences are seen.

Four different mutations were found in 60 family DNA samples analysed many of which did not have linkage confirmed due to the lack of DNA from key family members. These families had two or more cases of breast or breast/ovarian cancer. Figure 3.34.a shows the SSCP gel showing a band shift in exon 20 of the index case from pedigree Edin.BR1 relative to control samples. Figure 3.34.b shows the sequence of the sense strand of this exon from the index case revealing a C insertion at nucleotide position 5382 in exon 20 (5382 ins C>ter 1892), and the normal sequence of a control individual. The mutation was confirmed in the other strand by sequencing using the antisense primer. This mutation results in premature stop at base 1892 resulting in a shortened protein. This mutation has been reported as one of the most common mutations found in unrelated families with early onset breast /ovarian cancers throught the world. Figure 3.35.a shows a portion of the SSCP gel showing the band shift relative to control samples in exon 2 of the index case from pedigree CRC 3550. Figure 3.35.b shows the mutant sequence from the sense strand revealing an AG deletion at position 185 in the gene(185 del AG>ter 39). The normal sequence of this region is also shown. This results in premature termination of the protein at position 39 and hence a shortened protein. Once again this mutation has been reported in other families worldwide but in most cases

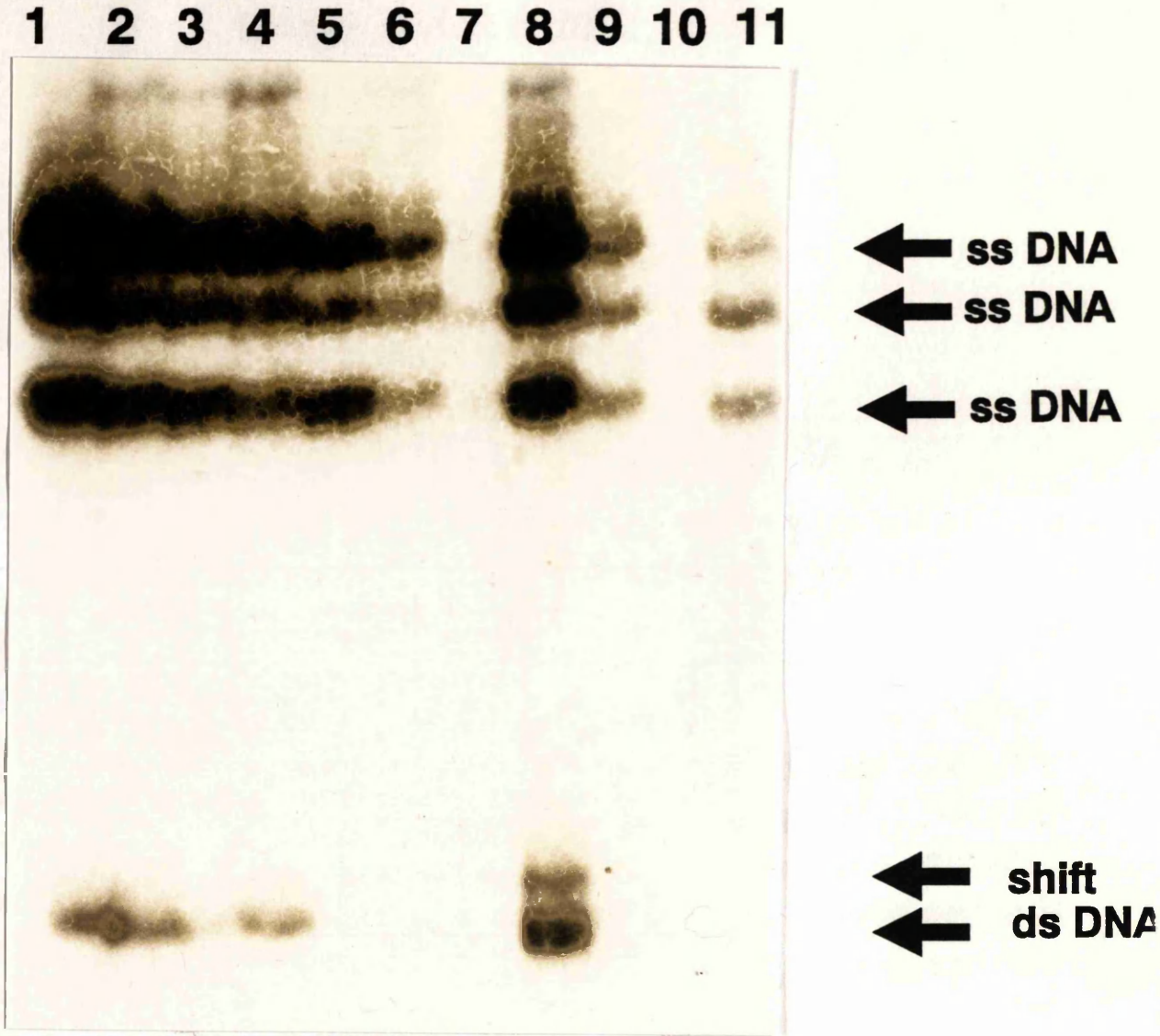
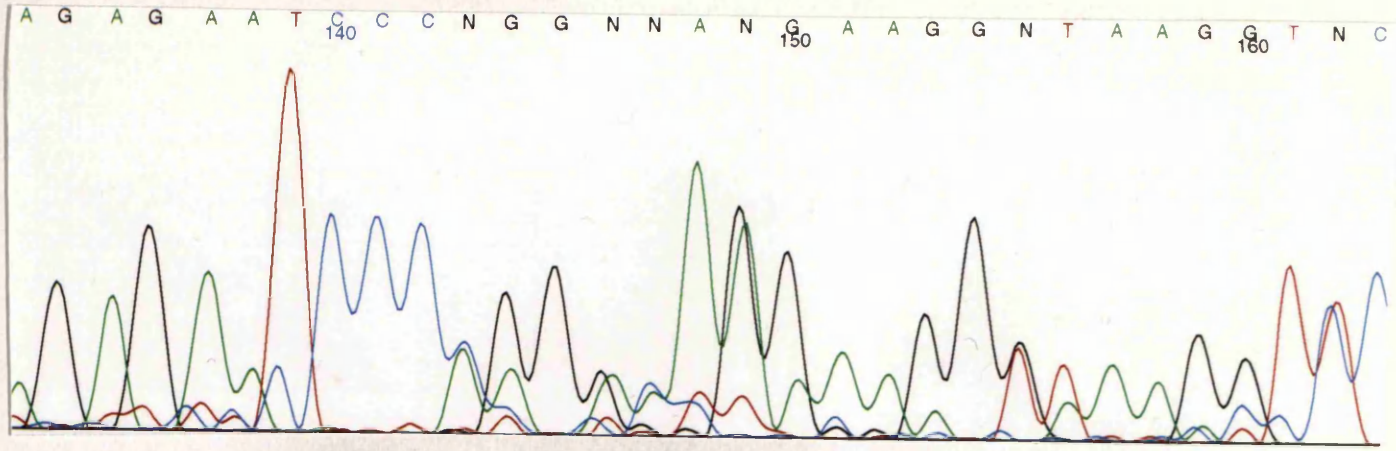


Figure 3.34 a : Autoradiograph showing part of a gel showing results of SSCP analysis of exon 20 of DNA from breast/ovarian cancer families. No band shift was seen in the single-stranded (ss) DNA but a band due to a heteroduplex was seen above the double-stranded (ds) DNA in lane 8. The sequence of this exon from this individual is shown in figure 3.34.b. The blank lanes represent PCR reactions which have failed.

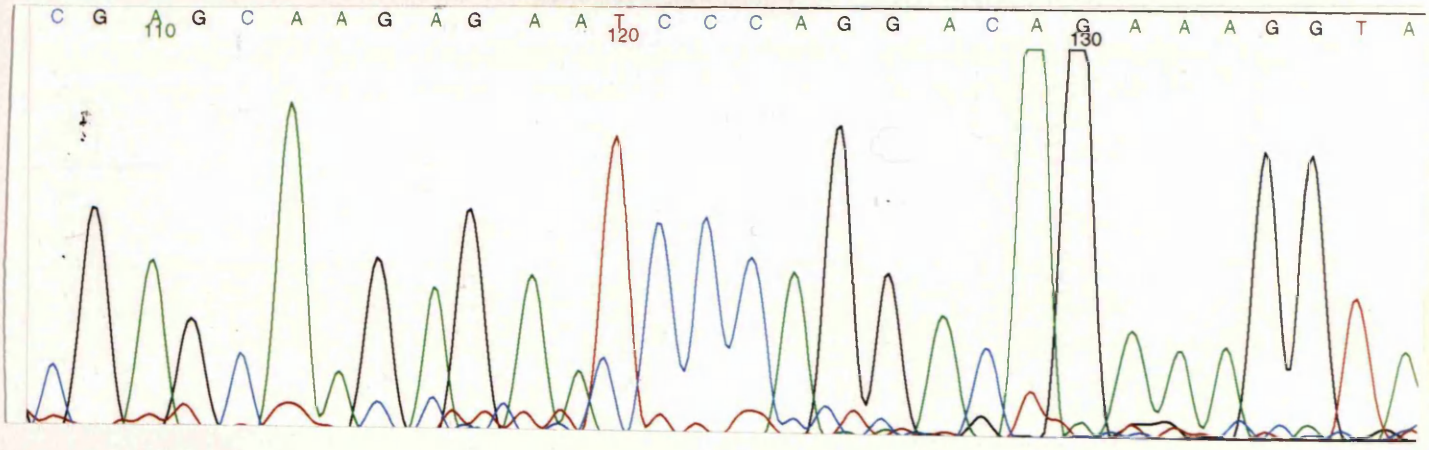
Figure 3.34.b : Part of a chromatogram from an automated sequencer, showing part of the sequence of exon 20 from an individual from a family with inherited breast/ovarian cancer. The sequence of the sense strand from the affected individual (A) and from a control sample (B) is shown and the position of the C insertion is indicated by an arrow in the affected individual. The position of the insertion is also indicated in the control sample although no insertion is present. The normal and mutant sequences are also shown.

C insertion in mutant



A

C insertion in mutant



B

sense normal sequence : AGAATCCCAGGACA

sense mutant sequence : AGAATCCCCAGGACA

Heterozygote : **************

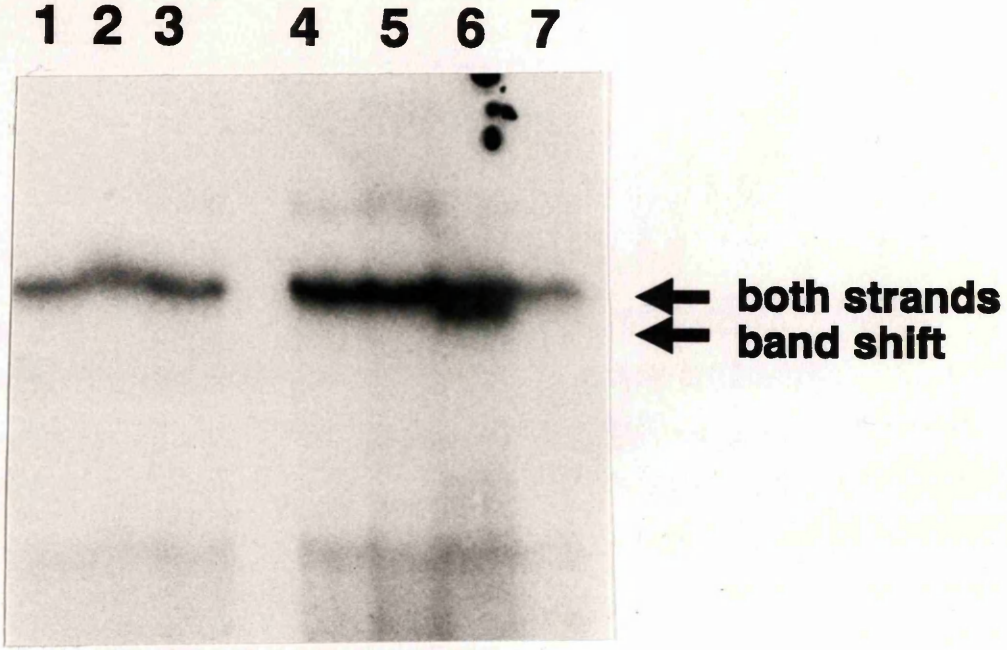
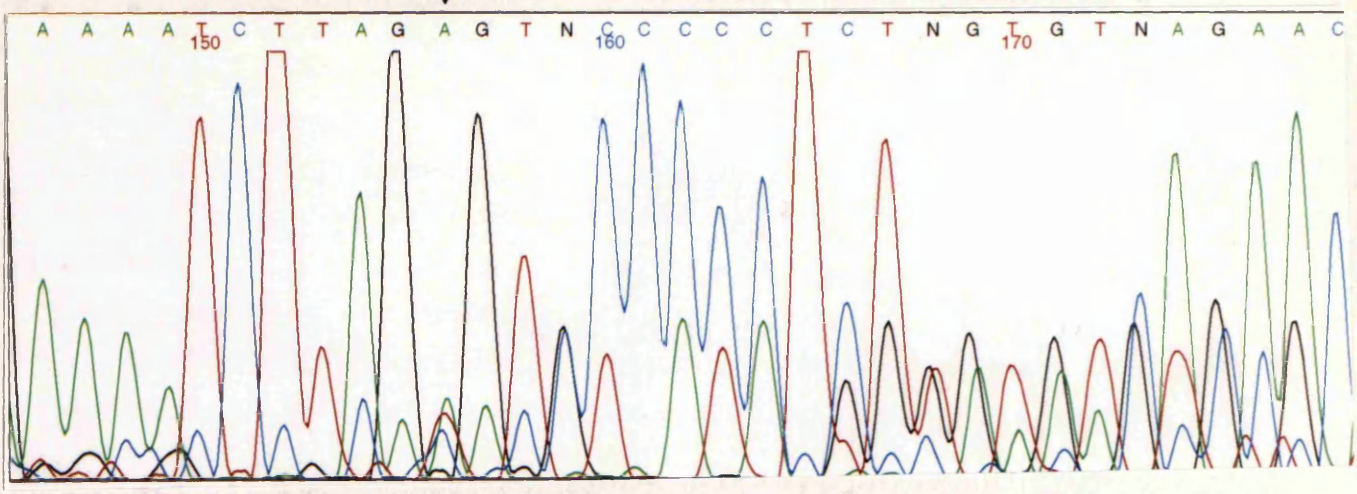


Figure 3.35.a. : Autoradiograph of part of a gel showing analysis of exon 2 DNA from breast /ovarian cancer families. The two strands do not separate but a band shift was seen in lane 6 relative to the normal DNA samples(lanes 1,2,3,4,5 and 7). The sequence of this mutation is shown in figure 3.35.b

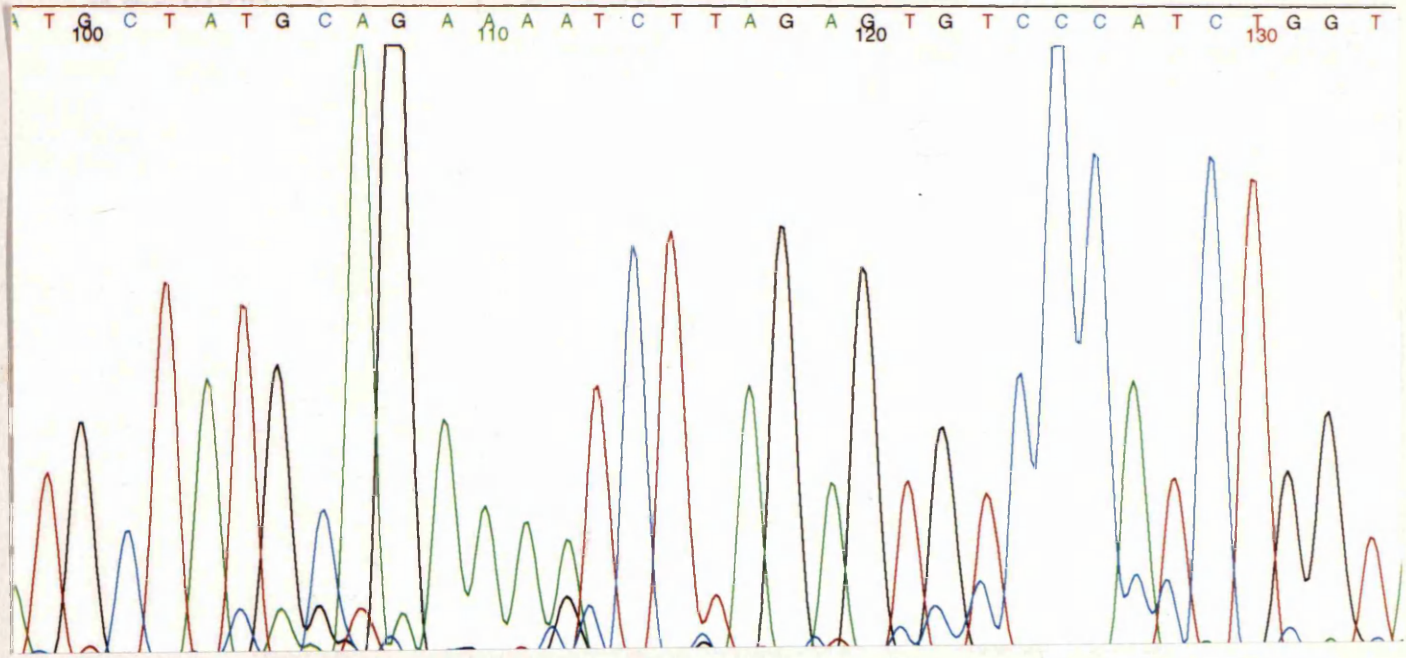
Figure 3.35.b : Part of a chromatogram from an automated sequencer, showing part of the sequence of exon 2 from an individual from a family with inherited breast/ovarian cancer. The sequence of the sense strand from the affected individual (A) and from a control sample (B) is shown and the position of the AG deletion is indicated by an arrow in the affected individual. The position of the deletion is also indicated in the control sample although no deletion is present. The normal and mutant sequences are also shown.

AG del in mutant



A

AG del in mutant



B

sense normal sequence : AAAATCTTAGAGTGTCCC
sense mutant sequence : AAAATCTTAGTGTCCC
Heterozygote : *****************

these mutations are present in families which can be traced back to the Ashkanasi Jewish population and hence a founder effect may have occured with this mutation in these families coming from one individual. No information is available as to the linkage of this family to the others but more detailed geneology is being carried out. Figure 3.36.a shows a portion of the SSCP gel showing the band shift relative to control samples in exon 2 of the index case from pedigree GLA. br1. Figure 3.36.b shows the mutant and normal sequences. Both strands of the mutant sequence are shown. This reveals an A to G substitution at base 180 which results in an Ile to Val amino acid substitution. The position of the substitution was analysed and was found to be present at the second base of the first RING finger motif and hence despite no change in the charge of the protein due to the amino acid substitution, the change at this position may affect the stucture of the RING Finger. The possibility remains that this change may be a polymorphism, and hence be functionally insignificant, but it was not found in any of the other 60 individuals analysed and untill further information is available as to the nature and function of the protein encoded by *BRCA1* , it is impossible to understand the importance of this mutation. Figure 3.37. a shows a portion of the SSCP gel showing the band shift relative to control samples in exon 11j of the index case from pedigrees Edin.br2 and Edin.br12. Figure 3.37.b shows a portion of the SSCP gel showing band shifts in the DNA of the index cases from pedigrees GLA475(lane 2) and Gla12343(lane 3). The sequence of the antisense strand of these mutations relative to the normal are shown in figure 3.37.c and shows a TT deletion in the antisense strand(AA in the sense strand) at nucleotide position 2800 which results in a premature stop at position 901(2800 del AA>ter 901), leading to a shortened protein. This mutation was previously described in one other American family (Friedman et al 1994). The discovery of the change in four Scottish families, may indicate the presence of a common Scottish mutation which is important from a diagnostic point of view. These families are at present being investigated to discover if they are related and if indeed they are related to the original American family to establish if the mutation comes from one common ancestor or if this region of the gene is specifically prone to mutation.

Several polymorphisms were also detected and the SSCP gels for these are shown in figure 3.38. a-c. The sequence of these polymorphisms in shown in figure 3.39.a-c A G to A substitution was found in intron 16

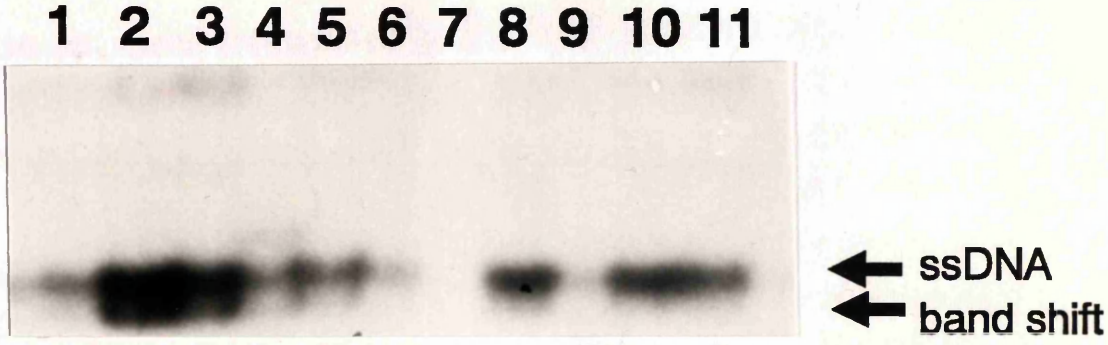
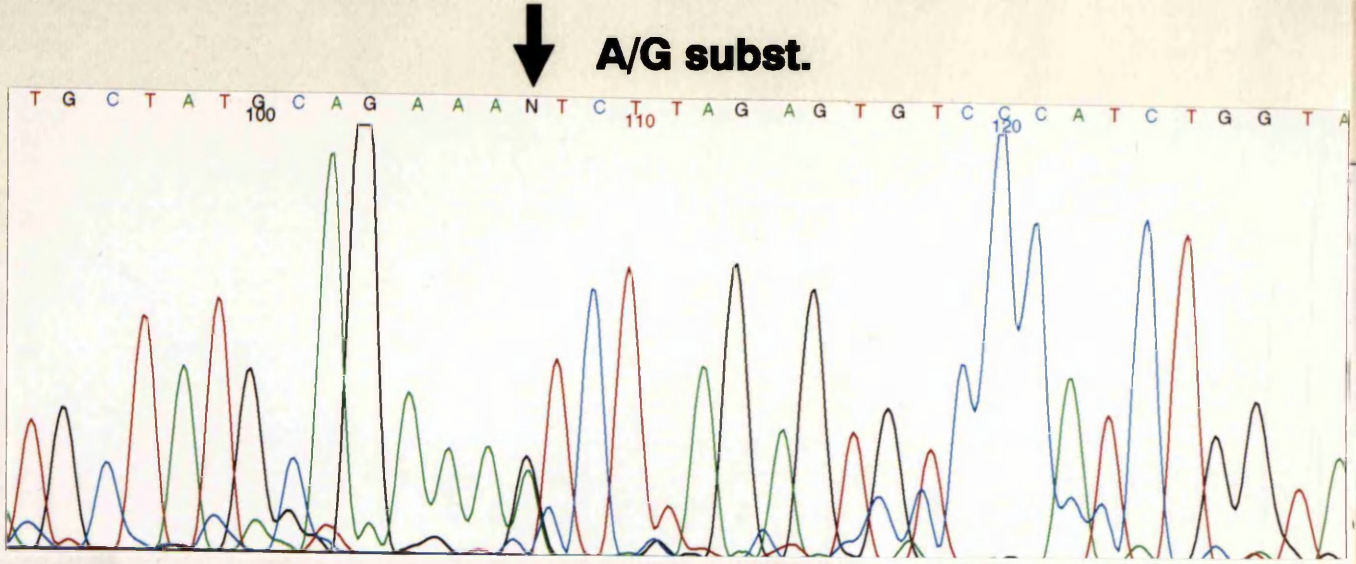
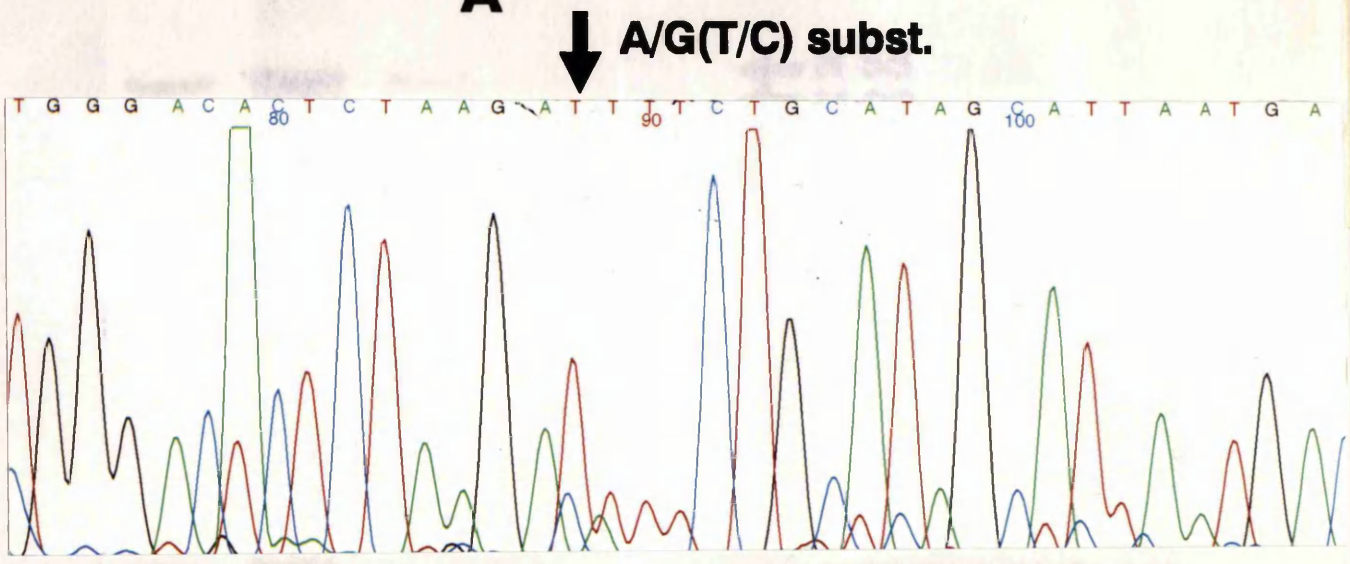


Figure 3.36.a : Autoradiograph of part of a gel showing results of SSCP analysis on exon 2 of DNA from breast/ovarian cancer families. The single-strands do not separate and the shifted band is indicated by an arrow. The band shift is shown in lane 2 with spillover of the mutant product into lane 3. The sequence o the shifted band is shown in figure 3.36.b.

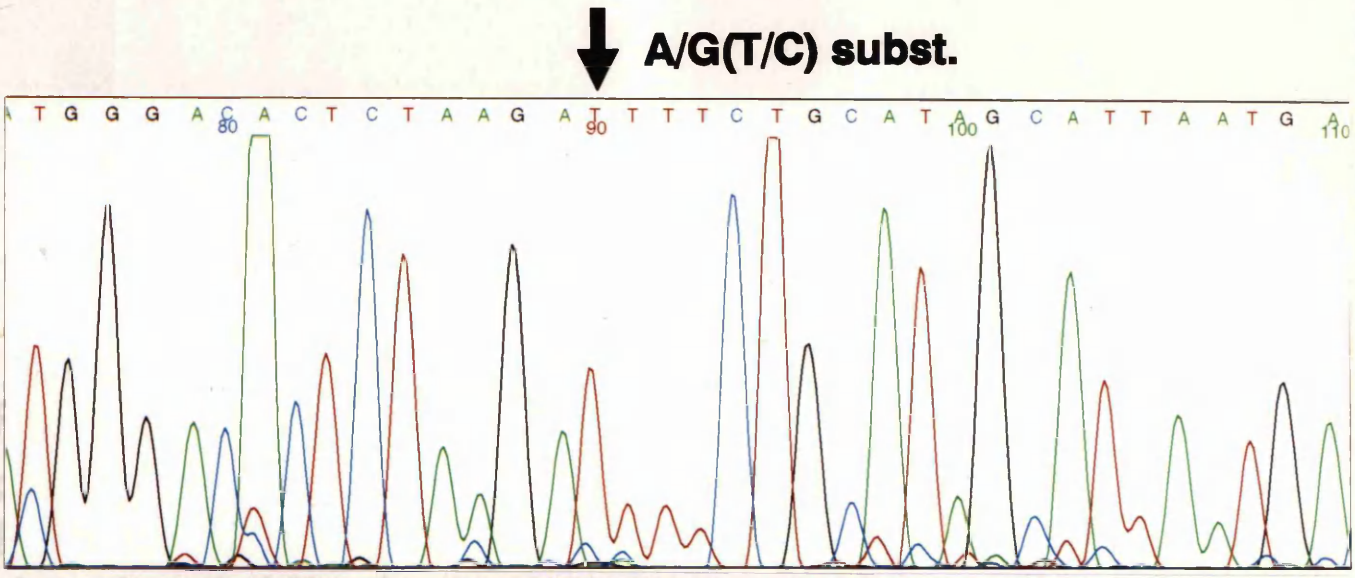
Figure 3.36.b : Part of a chromatogram from an automated sequencer, showing part of a sequence of exon 2 from an individual from a family with inherited breast/ovarian cancer. The sequence of the sense and antisense strand from the affected individual (A and B respectively) and the sequence of the antisense strand from a normal individual is shown (C). The position of the A-G substitution is indicated by arrows. The normal and mutant sequences are also shown.



A



B



C

sense normal : TGCAGAAAATCTTAG

sense mutant : TGCAGAAAGTCTTAG

heterozygote : A/G

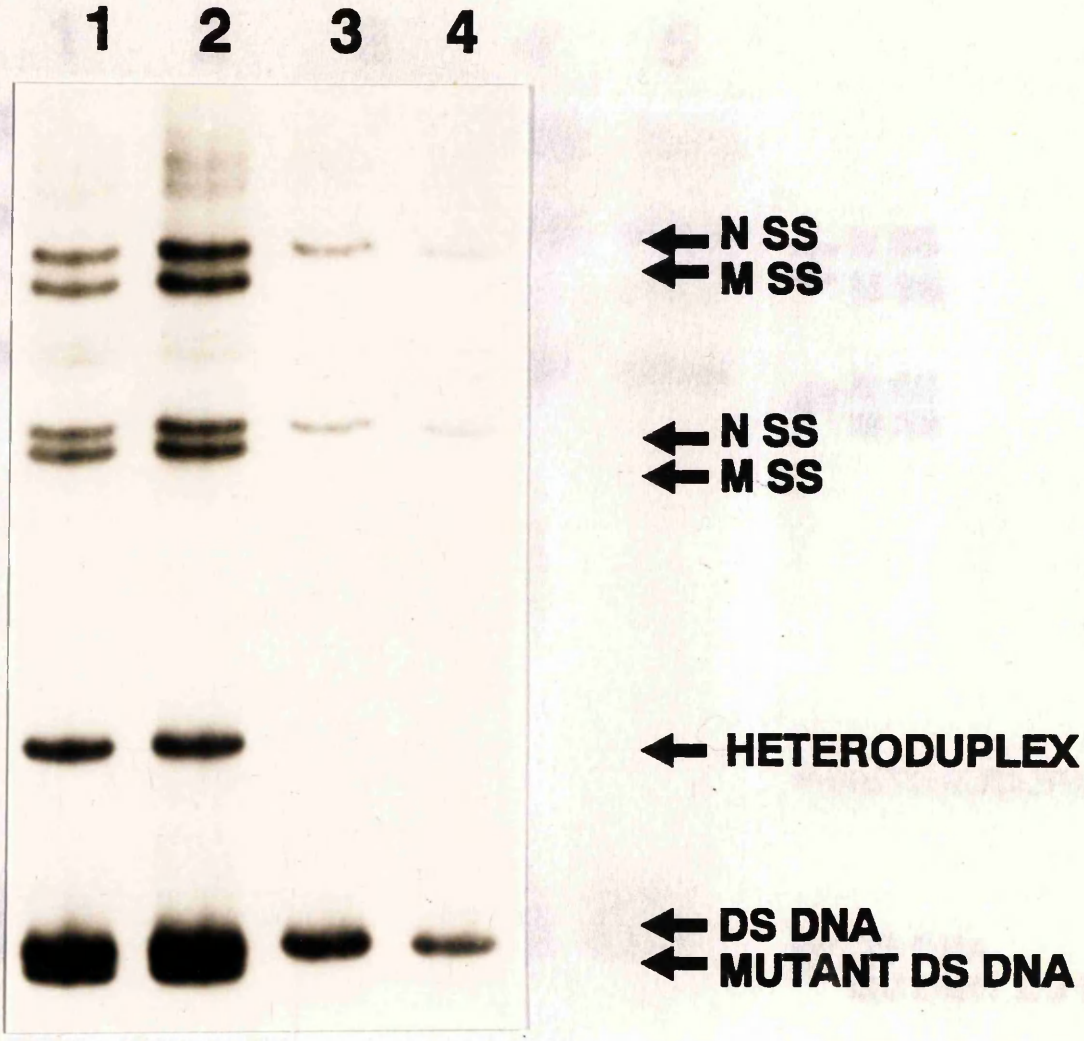


Figure 3.37.a : Autoradiograph of part of a gel showing results of SSCP analysis on exon 11 of DNA from breast/ovarian cancer families. Lanes 3 and 4 show normal exons while lanes 1 and 2 show band shifts due to mutant sequences present in two families, Edin.br2(lane 1) and Edin.br12(lane 2). The normal pattern of separation from normal DNA samples is shown in lanes 3 and 4. The sequence of the mutant bands is shown in figure 3.37.c.

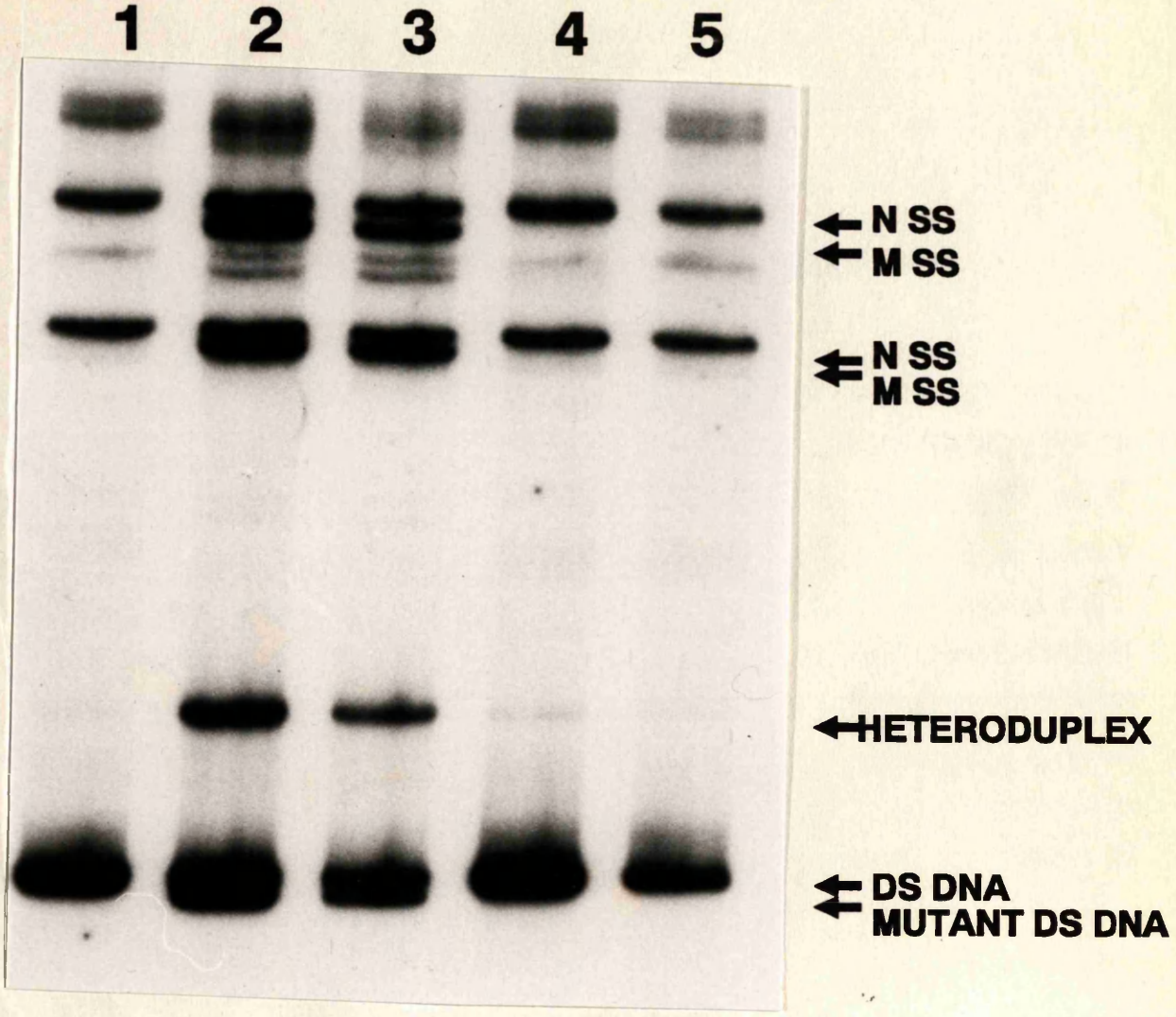
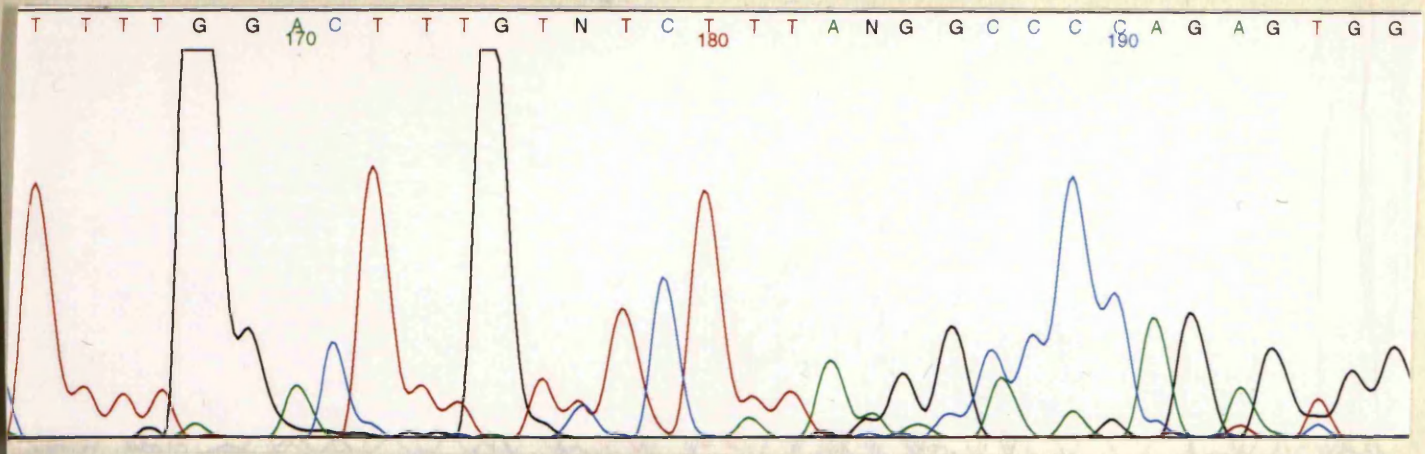


Figure 3.37.b: Autoradiograph of part of a gel showing results of SSCP analysis on exon 11 of DNA from breast/ovarian cancer families. Lanes 1,4 and 5 show the pattern from normal exons, while lanes 2 and 3 show the mutant pattern from DNA from pedigrees Gla 475 and Gla 12343. Normal bands are indicated by N and the mutant bands are indicated by M. The sequence of the mutant exons are shown in figure 3.37.c.

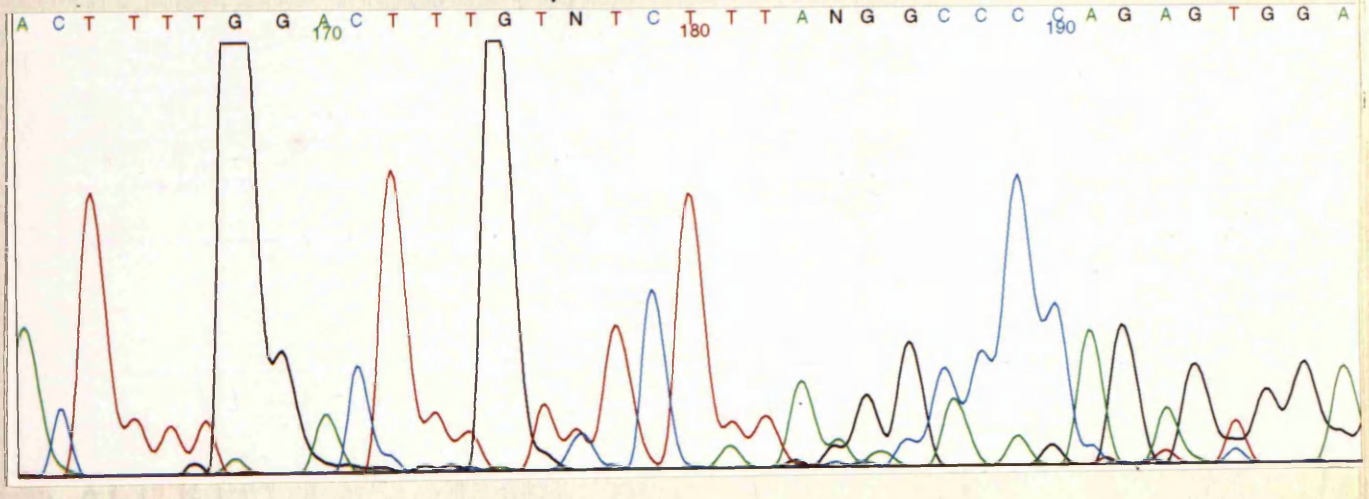
Figure 3.37.c : Part of a chromatogram from an automated sequencer, showing part of the sequence of exon 11 from four individuals from families with inherited breast/ovarian cancer. The pedigrees of these families are given in the text. The sequence of the antisense strand from the affected individuals (A - D) and from a control sample (E) is shown and the position of the TT deletion is indicated by an arrow in the affected individuals. The position of the deletion is also indicated in the control sample although no deletion is present. The normal and mutant sequences are also shown.

TT del in mutant



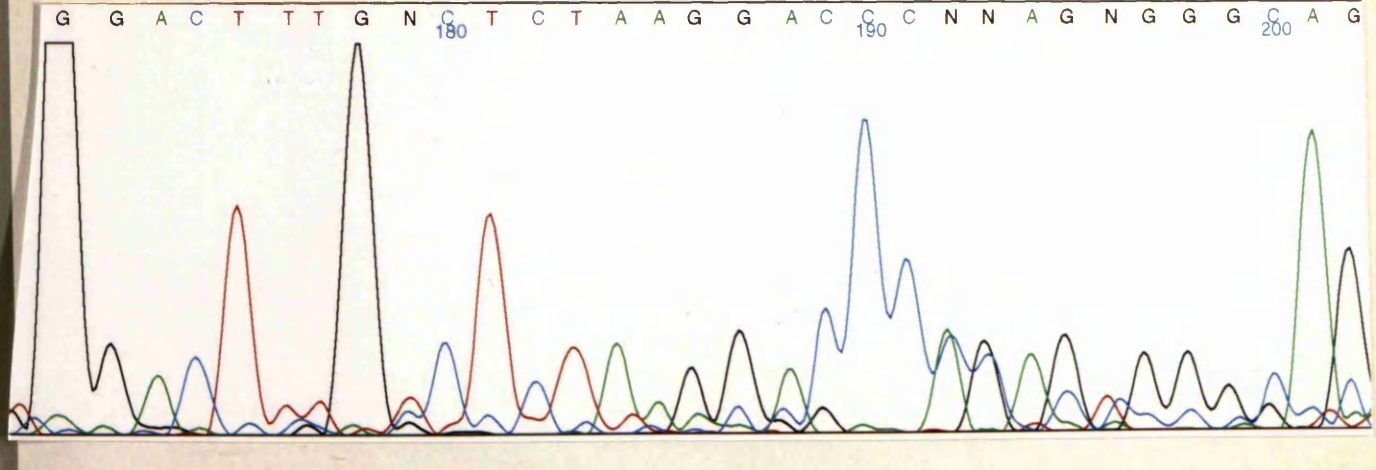
A

TT del in mutant



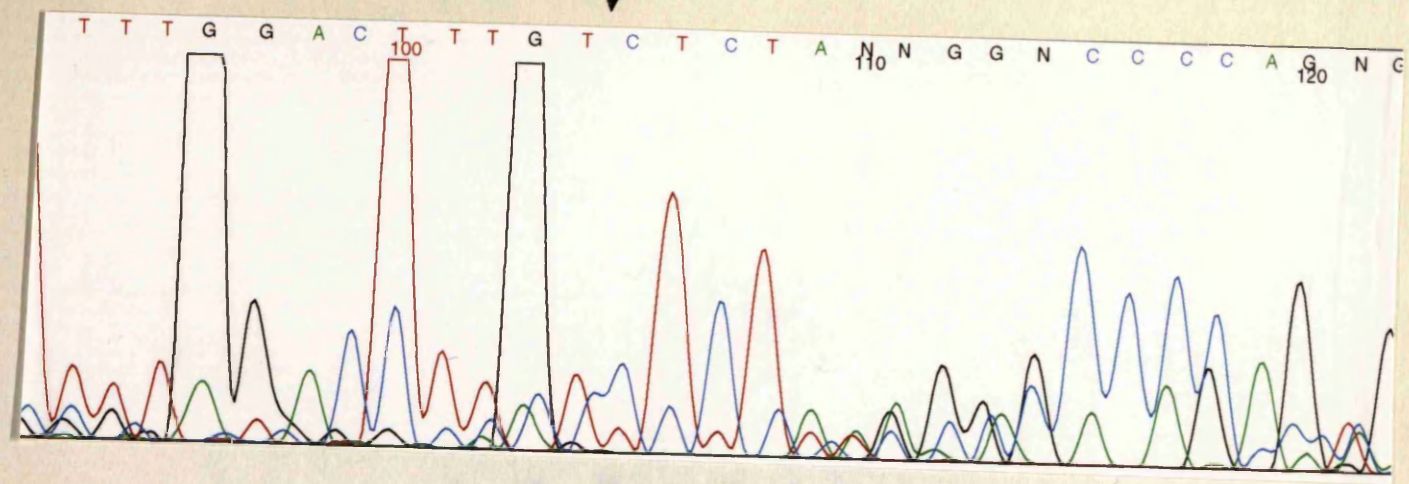
B

TT del in mutant



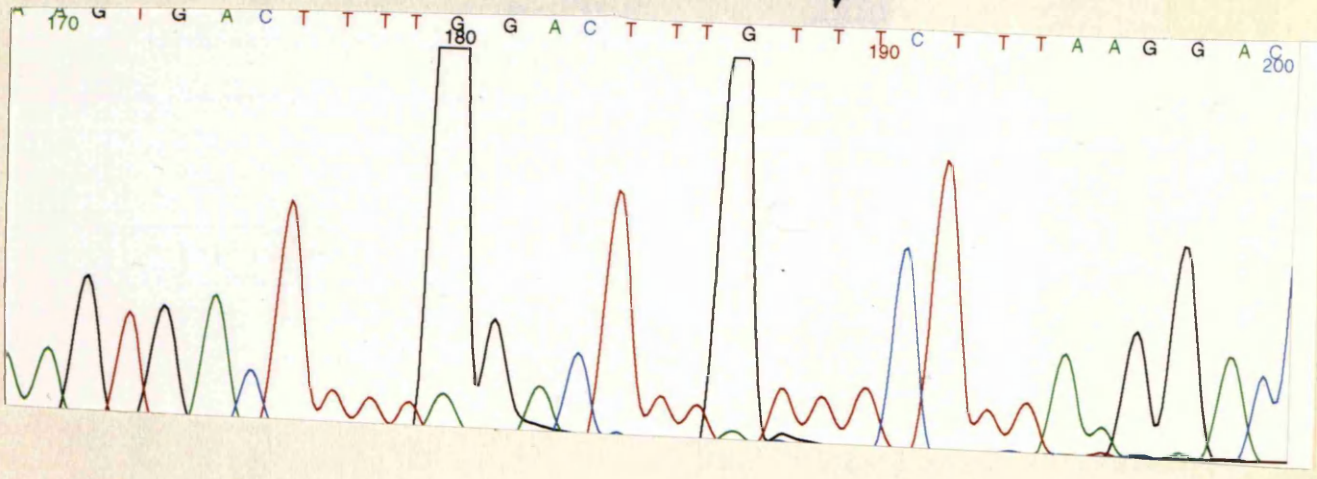
C

TT del in mutant



D

TT del in mutant



E

antisense normal sequence : TTGGACTTTGTTTCTTT

antisense mutant sequence : TTGGACTTTGTCTTTAA

Heterozygote : **********

Figure 3.38.a : Autoradiograph of part of a gel showing results of SSCP analysis of intron 16 DNA from breast/ovarian cancer families. Lanes 2 and 6 show the shifted pattern showing a polymorphism, the sequence of which is shown in figure 3.39.a. No homozygotes for the polymorphism were seen.

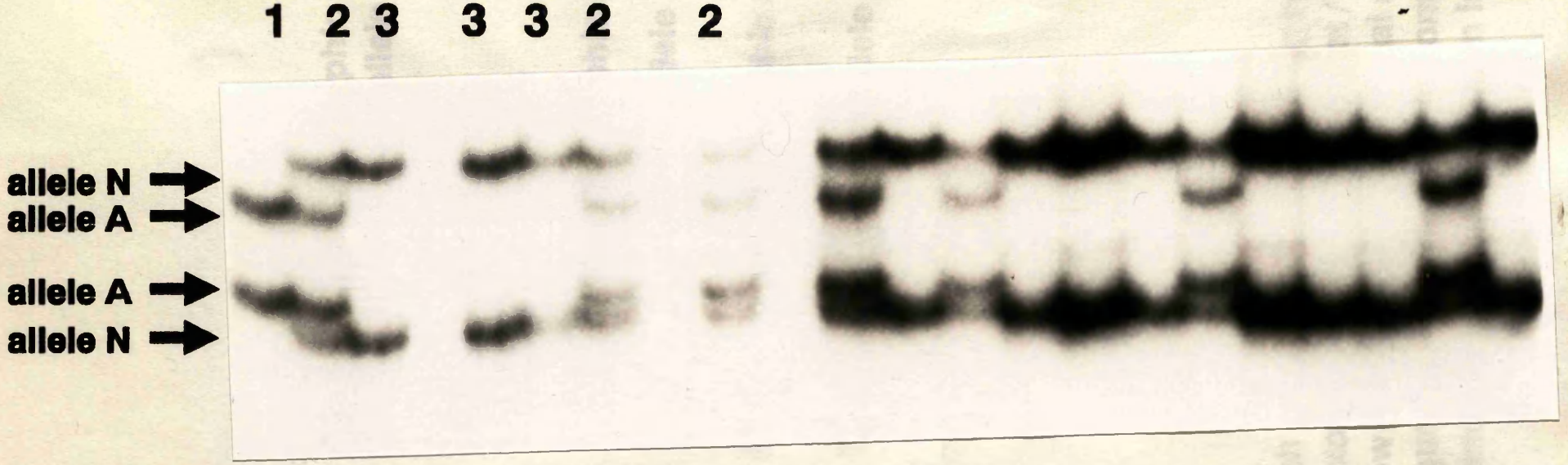


Figure 3.38.b: Autoradiograph showing results of SSCP analysis on exon 13 of familial breast/ovarian cancer DNA families. The band shift represents the presence of a polymorphism in the coding region of exon 13, with lanes 1 showing the homozygous polymorphic sequence, lanes 3 showing the homozygous normal sequence and lanes 2 the heterozygous sequence showing both alleles. The normal alleles are indicated by N and the affected alleles are indicated by A. The blank lanes are due to PCR failure in those particular samples. The sequence of this polymorphism is shown in figure 3.39.b

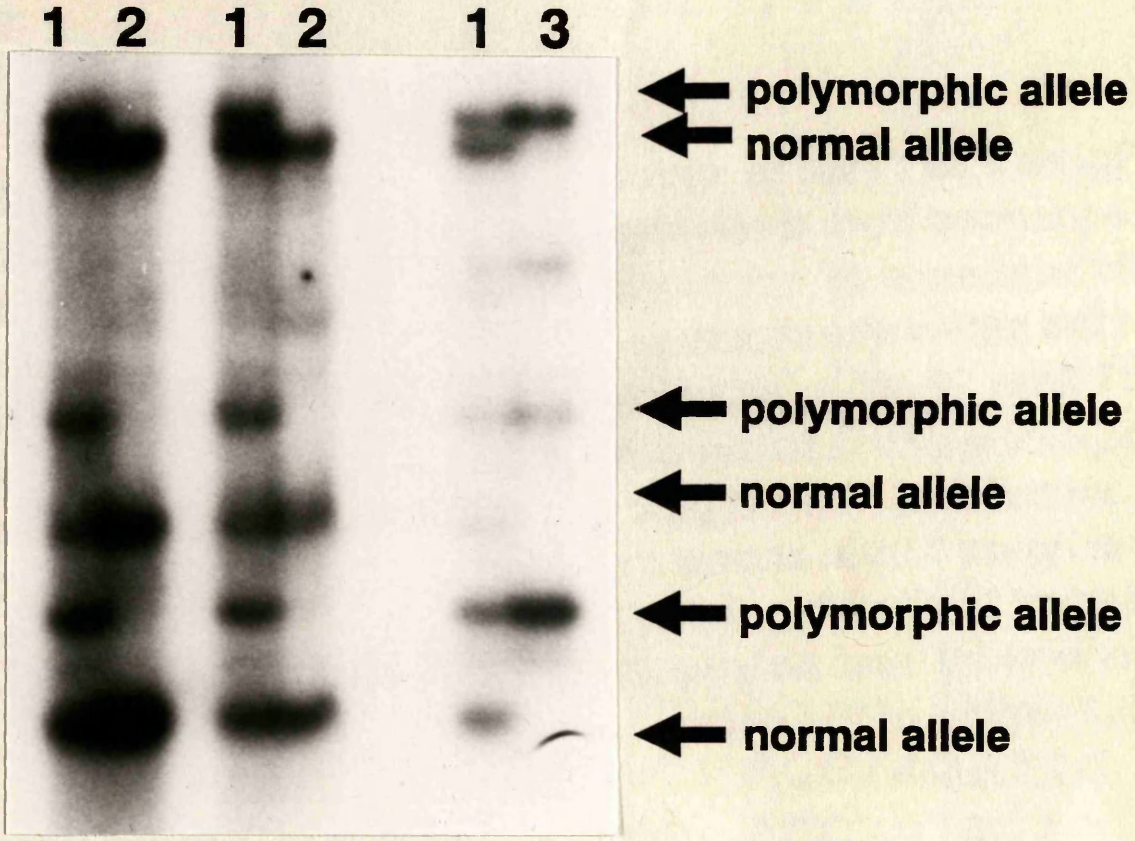
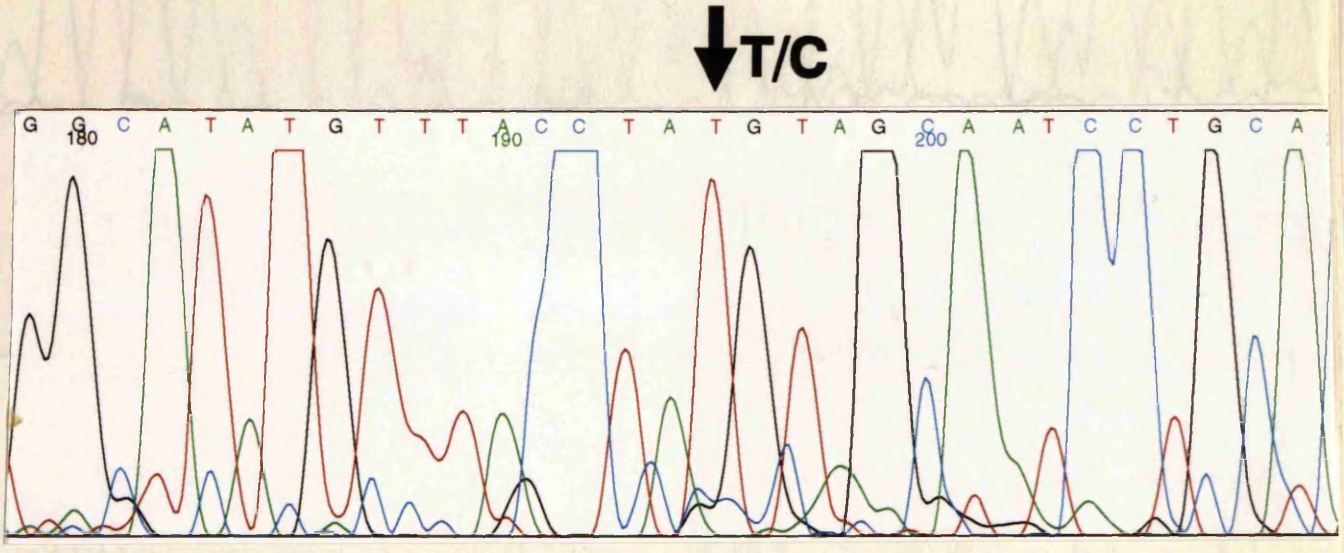


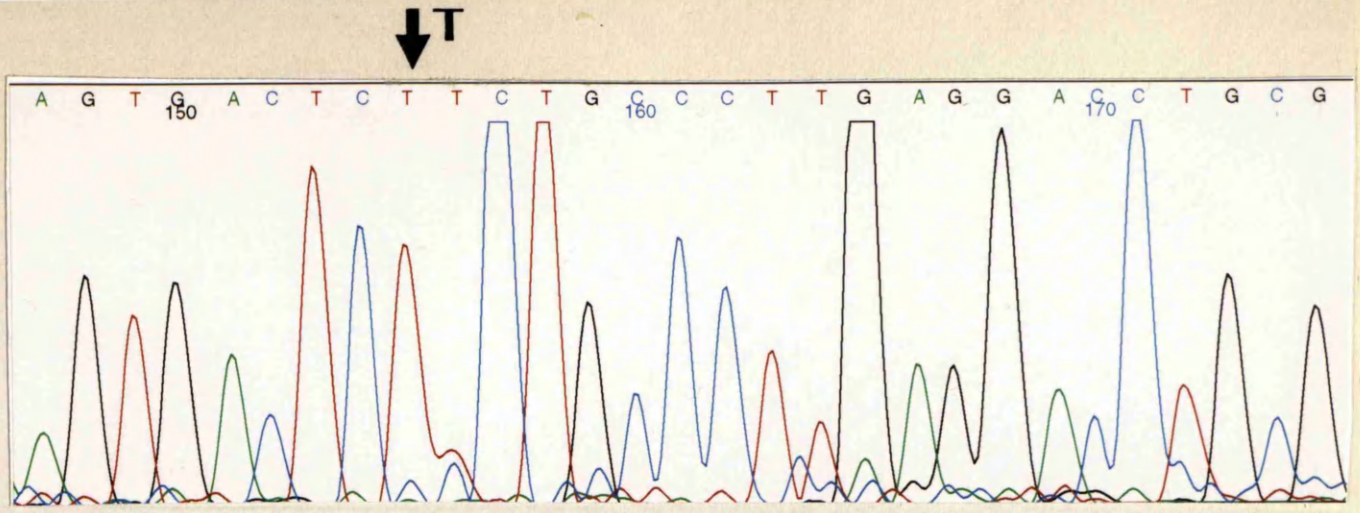
Figure 3.38.c : Autoradiograph of part of a gel showing results of SSCP analysis of part of exon 11 of DNA from breast / ovarian cancer families. Lanes 2 show the homozygous normal sequence, lanes 1 the heterozygous sequence and lanes 3 the homozygous mutant sequence. The sequence of this polymorphism is shown in figure 3.39.c.

Figure 3.39 : Part of chromatograms showing part of the sequences from three regions of the *BRCA1* gene showing the sequence of polymorphisms. Figure 3.39. a shows part of the antisense strand sequence of the A/G polymorphism in intron 16 of a heterozygote, figure 3.39. b shows the sequence of the sense strand of exon 13 from a heterozygote, a normal and homozygote for an intra-exonic C/T polymorphism, and figure 3.39.c shows the sequence the sense strand of part of exon 11 showing the C/T polymorphism from a heterozygote. The normal and polymorphic sequences are also given for each region of the gene.

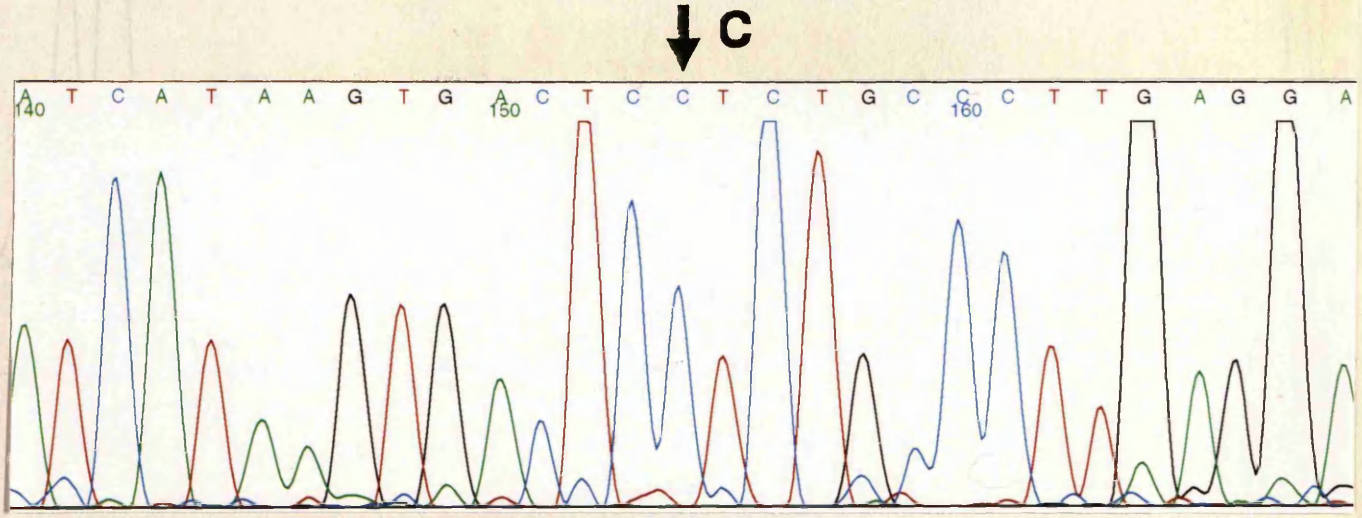


antisense normal sequence : ACCTACGTAG
antisense mutant sequence : ACCTATGTAG
heterozygote : T/C

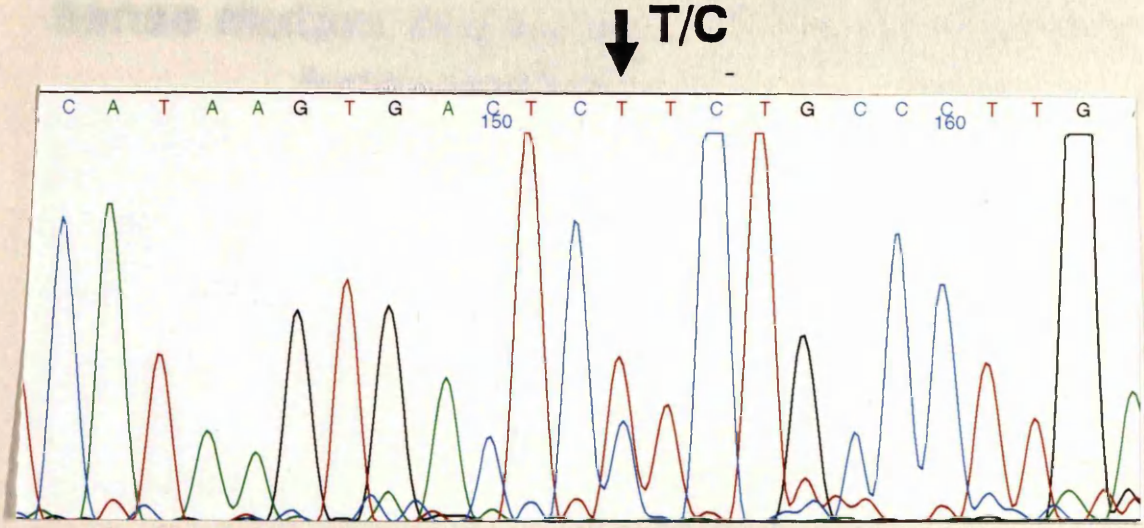
Figure 3.39.a



A normal sequence



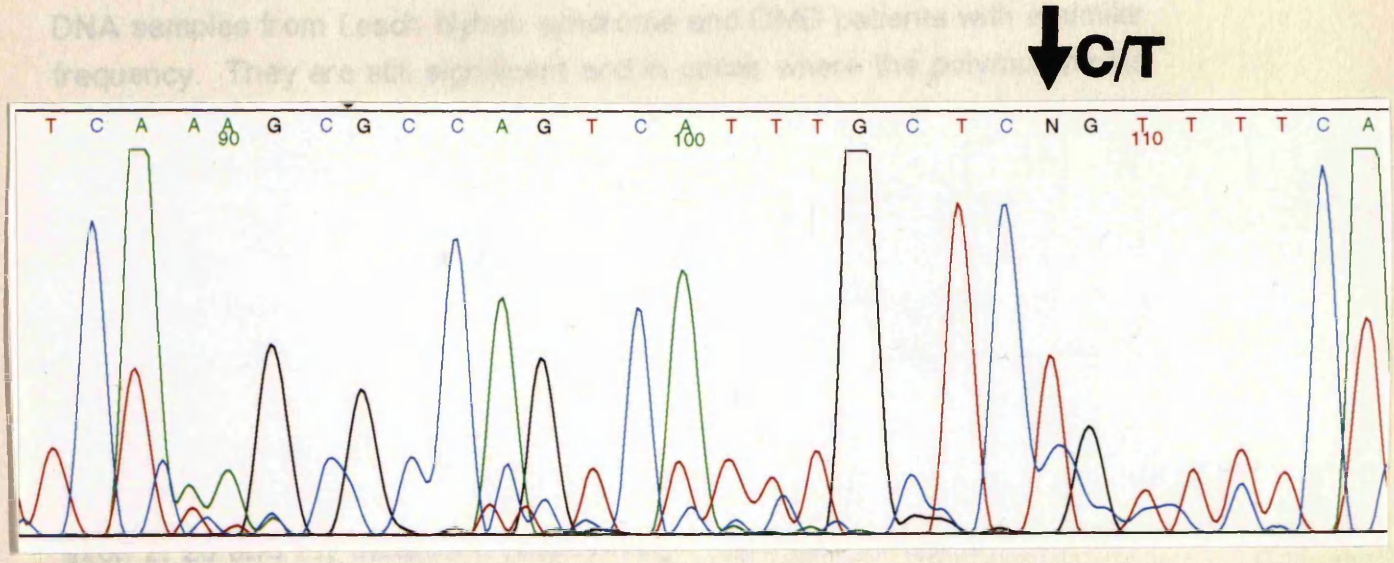
B polymorphism homozygote



C polymorphism heterozygote

sense normal sequence : GTGACTCTTCTGCCC
sense mutant sequence : GTGACTCCTCTGCCC
Heterozygote : C/T

Figure 3.39.b



sense normal sequence : TTTGCTCCGTTTT
sense mutant sequence : TTTGCTCTGTTTT
heterozygote : C/T

Figure 3.39.c

(fig.3.39.a), a T to C substituion in exon 13 at position 4427 (fig.3.39.b), resulting in no amino acid change and a C to T nucleotide substitution at position 2731, resulting in the substitution of a proline to a leucine(fig.3.39.c) was found in exon 11. These changes are known to be polymorphisms as they were found in many members of the families studied and also in control DNA samples from Lesch Nyhan syndrome and DMD patients with a similar frequency. They are still significant and in cases where the polymorphisms are informative, may help to trace the path of the affected chromosome through the families.

Once the family samples had been screened for mutations, the presumed sporadic breast and ovarian cancers were screened for the common mutations by ASO hybridisation. Oligonucleotides were designed which corresponded to the mutant sequences of the three common mutations in exon 2, 20 and 11. These oligonucleotides are described in 2.21. Figure 3.40.a-c shows the results of this screening using oligo,s for the exon 2, 20 and 11j mutations respectively. One autoradiograph for each oligo is shown although all the sporadic breast and ovarian tumours were screened. None of these mutations were found in the sporadic cancers.

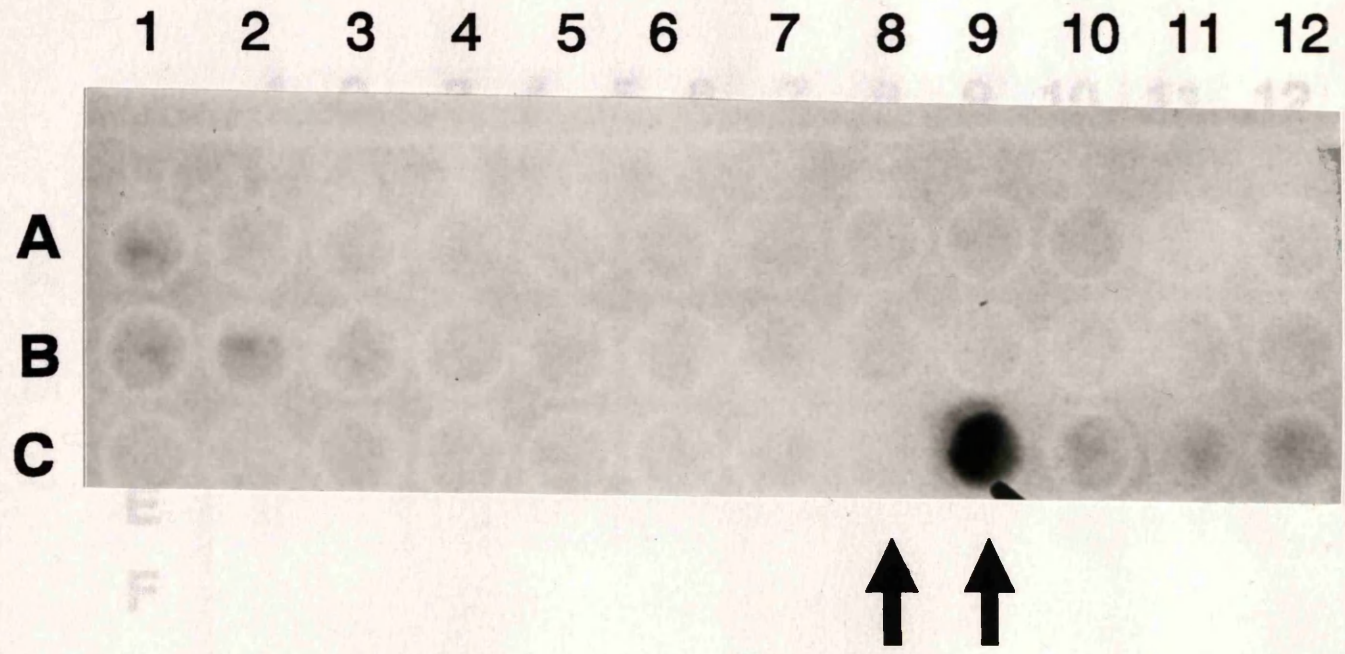


Figure 3.40 a: Autoradiograph showing results of an ASO hybridisation on 36 sporadic breast cancer DNA's using an oligo with the mutant sequence for the AG deletion in exon 2. The lanes contain exon 2 amplified DNA. A +ve control is shown in lane C9 and a -ve control in C8. These are indicated by arrows.

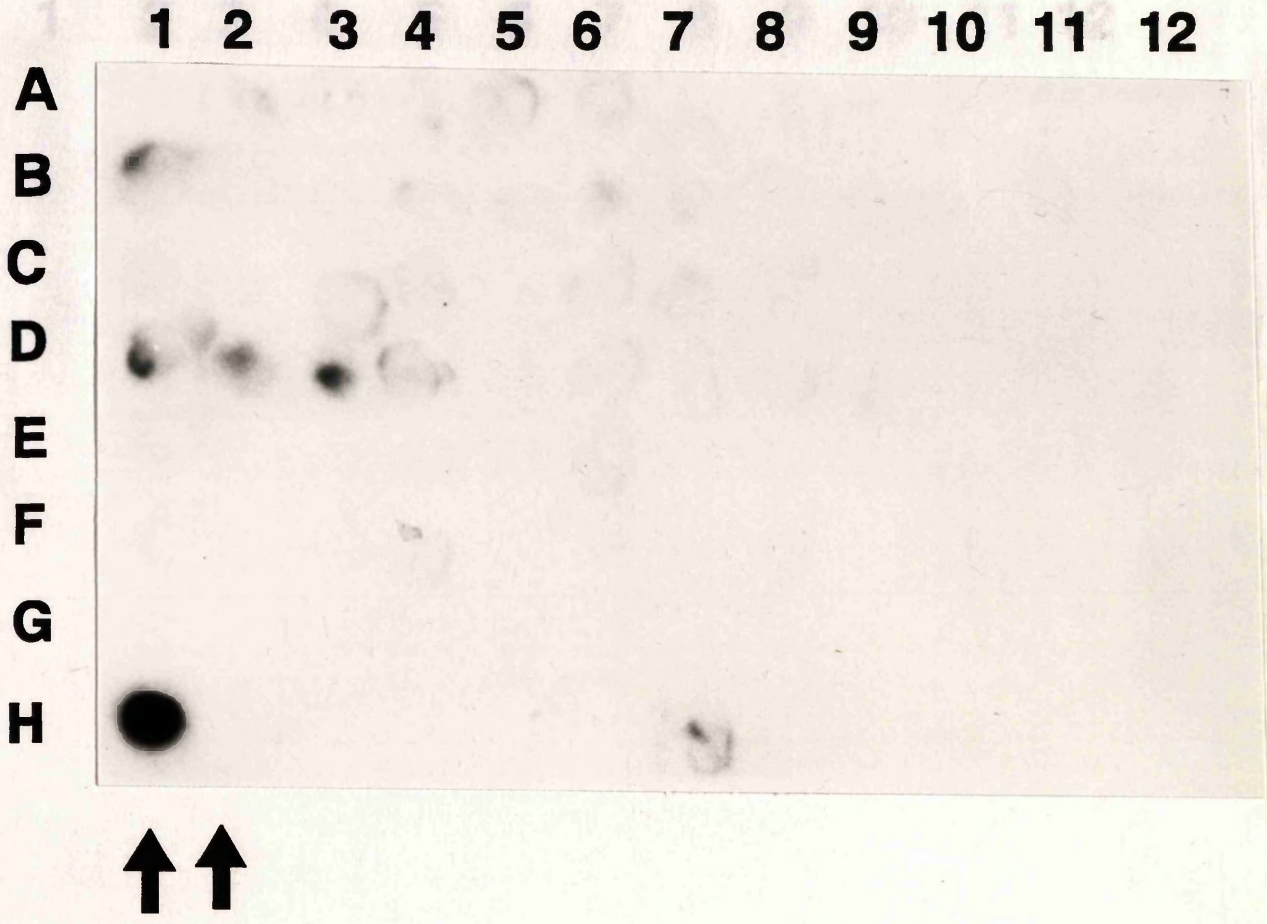


Figure 3.40.b : Autoradiograph showing results of an ASO hybridisation on 94 presumed sporadic breast cancer DNA's using an oligo with the mutant sequence with a C insertion in exon 20. A +ve control is shown in lane H1 and a -ve control in lane H2.

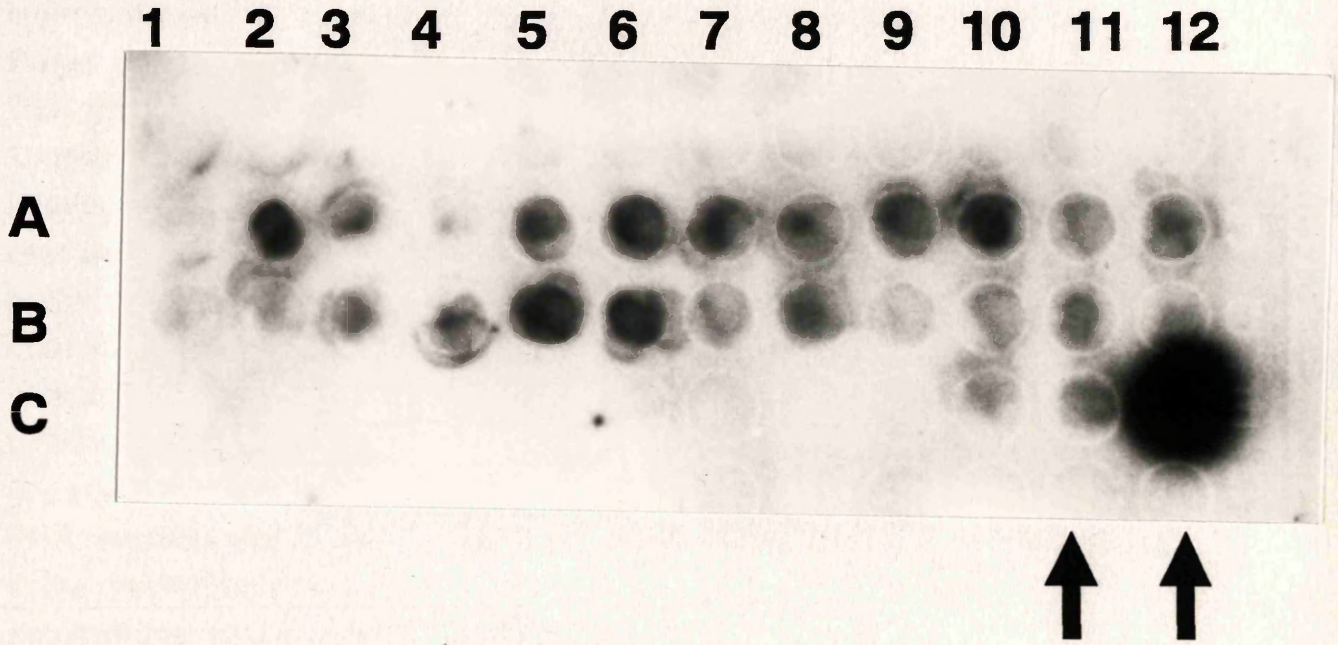


Figure 3.40.c : Autoradiograph showing results of an ASO hybridisation on 25 presumed sporadic breast cancer DNA's using an oligo with the mutant sequence of an AA deletion in exon 11. A +ve control is shown in lane C12 and a -ve control in lane C11. These are indicated by arrows.

CHAPTER 4

DISCUSSION

4.1 Optimisation and Comparison of the Screening Strategies.

4.1.1 Optimisation of the PCR Reaction.

The optimisation of the PCR reaction for the amplification of the individual exons of the *HGPRT* gene was complex and time consuming. Primer sequences for amplification of the individual exons in a multiplex PCR reaction were described by Gibbs et al. (1990), but when the conditions suggested in their study were modified for the amplification of the exons individually, no amplification was seen even after alteration of many of the reaction parameters. The reasons for this were several fold. Primarily, the buffer suggested was of a different composition to that supplied by the manufacturers of Taq polymerase (Boehringer) and appeared to be completely incompatible with amplification. The reasons for this are uncertain, but the addition of β-mercaptoethanol and $(NH_4)_2SO_4$ are unusual additions when compared with other buffers suggested for other PCR reactions and of particular note, are the absence of these components in the reaction buffers used in the amplification of the *HGPRT* exons in later publications using different primer sequences (Marcus et al., 1992, Steingrimsdottir et al., 1992, Lightfoot et al., 1992). Secondly, the concentration of dNTP,s used in the reaction was very high, with most protocols using lower concentrations of approximately 200μM of each dNTP rather than 1.5mM of each dNTP as suggested by Gibbs et al. The third problem was the unfortunate choice of exon 1 primers as the primer set to use for the optimisation of the PCR. The exon 1 region of the gene has a very GC-rich sequence and other groups later reported difficulty in amplification of this exon (Steingrimsdottir et al. 1992) and of the use of cDNA as the template for amplification of this particular exon. This was used due to the nature of the sequence which would be likely to later hinder direct sequencing from the PCR product (Marcus et al. 1992). The amplification of this exon was very problematic and despite eventual optimisation of the other exons using lower dNTP concentrations and the manufacturers buffer, the amplification of this exon consistently produced a 'ladder' of products on an agarose gel and the product of the correct size could not have been distinguished from other non-specific amplification products. As

RNA and consequently cDNA was not available for the majority of patients studied, analysis of exon 1 in patients with known mutations in that exon and application of molecular screening strategies to this exon were not possible, resulting in potential inability to locate molecular pathologies that may have been present in this part of the gene.

Most groups report the amplification of exons seven and eight together in a single amplification product. In this study further primer sets were made for this region which produced a smaller amplification product due to the large size of the product produced with the primer set used by Gibbs et al. (1990), which proved to be too large for use in some of the mutation screening strategies employed. Further, primer sets were also designed for the amplification of exons seven and eight individually for analysis of an index case (pedigree 10427) which originally appeared to have a deletion of exon seven/eight.

After optimisation of the annealing temperature to a sufficiently high level, non-specific products were generally not present. This was very important particularly in the formation of heteroduplexes where the presence of extra bands may have confused the interpretation of the results.

The oligonucleotide primers used in the PCR reaction also had to be of good quality or the PCR reaction failed. It had already been shown by other groups in the laboratory, that certain PCR reactions would not work if the primers used had not been purified after manufacture (Agapi Kataki, personal communication). This proved to be the case for the majority of the primer sets used in amplification of the *HGPRT* gene. Primers obtained from Oswell DNA services were HPLC purified and the primer sets made in the department on the ABI PCR mate oligonucleotide primer machine, were purified after deprotection by use of an ABI OPC column.

The quantity of DNA obtained by each amplification was not as high as concentrations obtained with other PCR amplifications including amplification of the *DMD* and *BRCA1* exons which shall be described later. This could be due to the fact that only 28 amplification cycles were carried out, but on increasing the total number of cycles to 30 no significant increase appeared to occur while 'eyeballing' the DNA concentration and a certain degree of smearing became apparent indicating that the quality of the DNA

was impaired. This lower yield of product per amplification reaction was taken into consideration when the amplification products were used in later manipulations.

All PCR reactions were overlaid with mineral oil. This was particularly important in reaction volumes of 50µl as failure to do so resulted in evaporation of liquid from the reaction mix and alteration of the concentration of the components resulting in non-amplification.

On several occasions during the course of these experiments, amplification of one or all of the exons failed despite original optimisation. Certain primer sets particularly those amplifying exons 2, 4, 6 and 7/8 seemed fairly robust, while other primer sets, such as exons 3 and 9, consistently failed to produce an amplification product after the primer sets were only a few months old. The reason for this is uncertain, but the most probable explanation is that the complementarity or physical properties of these particular primers were not ideal and that any degradation of the primers caused by continual freezing and thawing or by the build up of contaminants in the stock solutions, could not be tolerated during amplification. Primer sets which appeared less robust could usually be rescued by cleaning up of the primer by salt/ethanol precipitation, but failing this, a new primer set had to be manufactured which was costly and not always immediately possible. This hindered the analysis of molecular pathologies in exons 3 ,5 and 9 in particular.

Another reason for failure of individual PCR reactions was the quality and quantity of the DNA used. A large proportion of the DNA samples used as templates, were in the form of genomic DNA which had been extracted from old, frozen DNA and lymphoblastoid cell lines, some of which had been accidently thawed during transit and were presumed to be damaged. Several extracted DNA samples failed to allow amplification even from the most robust primer sets. As many of the index cases and family relatives were dead or unavailable for obtaining new samples, this factor severely hindered many of the linkage studies and direct mutation detection studies. Too much DNA in the reaction mix also hindered the amplification and this may have been due to the presence of higher concentration of impurities in the DNA which inhibited the activity of the Taq polymerase. If DNA samples which had previously been compatible with amplification began to be

problematic, this was usually an indication of degradation of the DNA or the build up of contaminants. This was the case on one particular occasion when many of the stock genomic DNA's would not amplify. This was found to be due to a build up of contamination in the samples to levels which affected both Taq polymerase and restriction enzymes and the contamination was found to be from the pipette tips which had picked up some form of contamination from the autoclave. After a phenol extraction and ethanol precipitation many of the DNA samples could be rescued.

If all the PCR reactions failed it was usually due to a fault with one of the reaction components and systematic changing of each of the PCR buffer, dNTP stock solution or Taq polymerase in the control PCR indicated the problem component. The most common problem component originally, was the dNTP mix particularly when the dNTP's were supplied by Boeringer. This was later less of a problem when the dNTP's were alliquoted on arrival. A further component which affected the reaction was the millipored distilled water. This water stock was used fresh each time a PCR reaction was set up and it was noted that if the pH of the ddH20 was higher or lower than expected or was not as pure as needed, the PCR reaction failed. This occured on several occasions but amplification was reinstated by use of bought water.

In contrast to the amplification of the *HGPRT* gene, amplification of the exons from the *DMD* and *BRCA1* genes were less problematic. The majority of the *DMD* exons amplified consistently which could have been due to the primers whose sequence had been published and which were used by most groups studying the gene, suggesting that the reaction conditions had been well optimised and tested. Further, initially the DNA available had been stored frozen since extraction and was in better condition and more plentiful than the DNA from the Lesch-Nyhan families. However only small quantities of DNA were available and once this was finished it was not possible to obtain other DNA samples as most of the index cases with DMD were dead.

The majority of primer sequences for amplification of the *BRCA1* gene were supplied by Miriad genetics, part of the group who discovered the gene and by Friedman et al., (1994). They suggested the best primer pairs and amplification parameters. A touchdown PCR was suggested which increases the specificity and quantity of amplification product obtained. Further, the DNA obtained was of good quality and this apppeared to

enhance the PCR. Several exons were difficult to amplify , including exon two which was the exon containing one of the common mutations. These primers did not produce a high concentration of product after amplification and after several freeze/thaw episodes or storage at 4^0C of a liquots, ceased to amplify the exon and new primers had to be prepared. This showed similarity to several exons of the *HGPRT* gene and may have been due to the sequence of the primer or to the sequence of the region it was amplifying.

The most influential factor in the amplification of sections of the genes studied, appeared to be the quality of the DNA being amplified and the degree of optimisation of primer sequences for the best amplification. Therefore the *HGPRT* gene amplification was problematic due to the poor quality of DNA available and the lack of research into better primers for the amplification of this gene. If these factors had been more closely considered and higher quantities of fresher better quality DNA were available, the PCR on this exon may have been less problematic.

4.1.2 Optimisation and Appraisal of Heteroduplex Detection on Hydrolink Gels,CCM and SSCP for the Detection of Pathologies in the *HGPRT* Gene.

Several mutation detection strategies including SSCP, CCM and heteroduplex detection were optimised and compared for their ability to detect known mutations in the *HGPRT* gene.

Most succesfully a modified PCR based heteroduplex detection assay using hydrolink gel electrophoresis, was optimised and evaluated against a panel of 10 different previously characterised mutations present in different exons of the *HGPRT* gene . After optimisation all 10 mutations were detected , taking into consideration unequal loading and that some heteroduplexes were clearer than others. Further, the technique was rapid, in this gene no false positive results were observed and the protocol proved to be simple, avoiding the use of toxic chemicals and radioactivity. However only 10 samples could be simultaneously examined which would limit its usefullness for analysis of larger genes.

Nagamine et al. (1989), reported the formation of heteroduplexes during PCR that could be visualised as slower moving bands in a gel. These heteroduplexes can be visualised as bands which migrate slower than their corresponding homoduplex bands due to a more open conformation which will result in retardation in the gel. Heteroduplexes are presumed to form during the later cycles of PCR probably because the DNA concentration increases to such a level that the complementary strands reanneal and outcompete the hybridisation of the primers with their template strand. Thus, heteroduplexes should be able to form during any PCR reaction where two non-homologous loci are being simultaneously amplified. It should therefore be possible to run a normal PCR product on a suitable gel matrix and visualise any heteroduplex bands, but in these experiments, modifications had to be made to the PCR reaction to maximise the formation of heteroduplexes and to provide the optimum conditions for obtaining clear results with no spurious or 'ghost' bands to confuse the interpretation. The optimum results were achieved by completely denaturing the duplexes by heating to 94^0C and then holding the reactions at 70^0C for up to 16 hours and directly loading the DNA onto the gel without cooling. To ensure a clean PCR product without the presence of 'ghost' bands, a two step PCR protocol was employed as suggested by Keen et al. (personnel communication) and later Inglehearn et al. (1992). Both suggest that the two step PCR coupled with the holding at such a high temperature minimise the formation of the extra 'ghost bands' which could be seen directly above the main product.

Before the advent of this project, the literature documented the application of this protocol for heteroduplex detection in autosomal single gene disorders such as cystic fibrosis, autosomal dominant retinitis pigmentosa and Tay-Sachs disease (Triggs-Raine and Gravel, 1990; Keen et al. 1991). Due to the X-linked nature of both HGPRT deficiency and DMD/BMD, two loci would not be present to be simultaneously amplified as there is only one copy of the X-chromosome in males. Therefore while this strategy could be employed for carrier detection in female relatives, in its original form, it would be of no use for identification of mutations in males. Control DNA was therefore added to the PCR reaction to create a potentially heterozygote state in males and this widened the application of heteroduplex detection to X- linked diseases. Since then other groups have used this technique for analysis of X-linked disease in particular DMD (Prior

et al. 1993). If the technique were to be used for the analysis of autosomal diseases no control DNA would need to be included.

Several matrices had previously been used for separation of heteroduplexes from homoduplexes. The early publications used polyacrylamide gels but the homo- and heteroduplexes in these experiments differed by four or more base pairs (Nagamine et al. 1989; Angliani et al. 1990; Triggs-Raine et al. 1990). Keen et al. (1991), introduced a novel non-denaturing gel matrix , hydrolink (AT Biochem) , as a more sensitive matrix for the separation of fragments differing by as little as one base-pair. This polymer had only been previously used by Keen et al. (1991)and its use had not previously been evaluated so it was therefore used in this study and other groups have successfully used this matrix and the modified polymer ,Hydrolink-MDE for the detection of a wide variety of mutations in several different genes (Ingleherrn et al., 1992; Prior et al., 1993). White et al., (1992), successfully utilised denaturing acrylamide gels to detect single base substitutions but this required the use of radioactive labelling to allow the detection of the smaller quantities of DNA that can be loaded on thinner acrylamide gels. One of the main advantages of hydrolink gels is the speed at which results can be obtained from a simple protocol that does not employ the used of toxic components such as radiation and for our purposes the hydrolink matrix serves this purpose better than an acrylamide one. Further, not all of the substitutions analysed by White et al., (1992) were detected by their methodology, while in this study all tested mutations were detected after optimisation .

The electrophoretic conditions used were important in the maximisation of the sensitivity of the protocol. Variation in the length and thickness of the gel used allowed detection of all 10 mutations with the optimum thickness of 1.5 mm allowing loading of a higher quantity of DNA without loss of resolution. The thicker gels were also easier to handle allowing easy staining of the gel negating the need for the use of radioactivity to detect smaller quantities of DNA on flimsier gels. The mutations present in larger amplification products required to be run through longer gels to allow separation of the different duplexes and as a rule any PCR products exceeding 1kb in length were analysed on a 20 x 30cm gel. Some mutations, such as the GT deletion in the family HGPRT Cheltenham (figure 3.24.), actually produced different patterns of separation when run on

longer gels compared to the shorter gels The speed and voltage at which the gel was run was also critical. Gels were run as slowly as possible to obtain maximum separation and at low voltages to prevent the gels from heating up and possibly causing disassociation of the heteroduplexes due to the unstable nature of mismatches.

The mutation HGPRT Marlow (C527T) was amplified in a PCR product which simultaneously amplified exons 7 and 8 in one PCR product. The original PCR product was 1533bp in length and despite optimisation, heteroduplexes could not be detected for this mutation. A second set of oligonucleotide primers were subsequently designed for amplification of this region in a product 433 bp in length. The mutation was then easily identified and a heteroduplex band seen. As heteroduplex bands were visualised when mutations in fragments 1278bp were analysed but not in a fragment size of 1533bp, it can be assumed that there is an upper limit to the size of fragment that can be analysed in this way and that this limit is somewhere between 1278bp and 1533bp. However most mutation detection techniques have this limitation in particular SSCP analysis, where sensitivity is maximum with fragment sizes below 200bp in size and decreases with every 100bp. An upper size limit of the levels witnessed with this heteroduplex detection in hydrolink gel protocol is therefore very useful and further increases the value of the technique.

Different patterns of separation were seen including single and doublet heteroduplex bands. Also apparent with some mutations was a thickening of the original heteroduplex band rather than the presence of a definite extra band . This could cause confusing results and indeed in some cases where the wells of the gel had not formed properly this thickening was seen in all the lanes of the gel. However it was usually clear when this had occured as more than one sample was affected and the use of more than one gel and one PCR product for each sample limited the likelihood of a false positive result. This however does highlight the importance of the need for experience to determine whether or not a band is real or an artefact and the use of the known mutations to analyse the effectiveness of this technique, allowed lessons to be learned before the protocol was used to look for uncharacterised mutations. Due to the nature of the gel matrix, on several occasions, such as with the family with the mutation HGPRT GLA 7292 the relative mobilities of the homo and heteroduplex bands vary across

the width of the gel as is seen in figure 3.25. This did not confuse the results as the presence of the extra bands was still clear and to ensure the validity of these results several different samples were run on several different gels.

A surprising feature of heteroduplex analysis that had been noted by several different groups as well as in the present study, is the apparent lack of correlation between type of mismatch and degree or pattern of separation. For example it would be expected that the 1-bp insertion present in exon 3 of the boy with the mutation HGPRT Codicote (T298) ,would result in a slower moving and hence more separated heteroduplex band than the HGPRT Banbury mutation present in exon three as a G-C substitution. However the degree of separation is almost identical. Further the mutation HGPRT Runcorn (T395C), which results from a T-C substitution gives one of the best patterns of separation which is similar to the separation obtained with the 2-bp deletion in exon three, HGPRT Cheltenham (GT 288-289). The reason for this is uncertain but it could be due to the degree of disruption of hydrogen bonding that is caused by the relative mutations causing more or less open conformations around the mismatched base or bases. White et al., (1992), investigated this possibility and concluded that the identities of the nucleotides adjacent to the mismatch had a substantial effect on the conformation of the mismatched region in terms of the hydrogen bonding effects and in terms of the effects on base stacking. In these experiments, it is difficult to make any conclusions as to the effect of the particular mutation on the degree of separation due to several factors including the differing sizes of the amplified fragments containing the mutation. As all mutations are detected in the study it is very difficult to speculate on the mechanisms at work.

There were two negative aspects to this protocol. Primarily , the difficulty in recording photographically the results obtained from the ethidium bromide staining of the hydrolink gels. Often the gels stained non-uniformly creating hazy gels which did not photograph well. When diagnostic results are being reported, one needs to have clear data available and even the use of radiolabelled products on longer thinner gels did not alleviate this problem as the heteroduplexes on these gels were difficult to distinguish. Secondly, the nature of the hydrolink gels and the desire to load as much sample as possible into the individual wells, limited the number of samples which could be run at one time. This hampered the use of this protocol for

the analysis of larger multiexonic genes such as the *BRCA1* and *DMD* genes in particular where large numbers of samples were to be analysed.

A protocol for detection of point mutations using the CCM strategy (Cotton et al. 1988), was established using two known mutations in the cystic fibrosis and *DMD* genes. These mutations caused a G-A mismatch and should result in cleavage by piperidine after modification with hydroxylamine.

While effective, this strategy proved to be problematic and time consuming and for optimisation, two strategies were employed to obtain cleavage of the CF G551D mutation, due to the lack of a cleavage using the original strategy suggested by Cotton et al. (1988). These differed primarily in the method of probe preparation and in the use of siliconised tubes for the reactions. The original protocol relied on radioactive labelling of the probe after PCR amplification and even after purification of the PCR product with geneclean to remove any components which could adversely affect the labelling reaction, the incorporation of label onto the probe was very low. Consequently, increased amounts of probe were used to set up heteroduplexes, thus requiring increased quantities of unlabelled test DNA. Despite achieving a visible signal resulting from the uncleaved probe a cleavage product was consistently absent.

Other factors were investigated including methodology for cleaning of the unlabelled test DNA, ratios of unlabelled test to labelled probe DNA, times of incubation with both hydroxylamine and piperidine and viability of stock solutions of chemicals, and solutions. However no cleavage occured even after variation of all of the variables. This was presumed to be due to vastly increased quantities of DNA used in order to achieve a signal, which was probably interfering with the chemistry of the reaction or inhibiting formation of sufficient quantities of heteroduplexes.

The second approach utilised two different methods of probe preparation, labelling of the PCR primers before amplification and inclusion of a radioactive nucleotide in the PCR reaction to achieve labelling of the probe along its length. Both of these methods produced better labelling of the probe but slightly increased levels of probe still had to be used in order to obtain a clear signal. Cleavage was obtained with the CF mutation using

siliconised Sarstedt tubes as suggested by Howells et al. (1990), and a probe labelled along its length, produced three bands , the uncleaved band and the two parts of the amplification product after cleavage. The *DMD* mutation was subsequently detected using 5'-end labelled probe and only one cleavage band was seen as expected due to the fact that only one end of the probe was labelled.

Other groups utilised both of these methods successfully and suggested the inclusion of only one 5-prime labelled primer to give accurate information as to the exact positioning of the mutant gene depending on the size of the cleavage product. Although helpful this approach was neither feasible nor particularly necessary in these studies due to the low level of labelling which was later observed with the *HGPRT* gene. As any suspicious exons were to be sequenced fully in the search for the molecular pathologies, the exact position of the mutation was not necessary particularly since this caused a decreased signal and could have led to the non-identification of a cleavage event due to the faint appearance of the cleaved product, as two primers needed to be labelled to ensure a signal of reasonable intensity.

The autoradiographs still had to be exposed to the gels for one to two weeks for a clear signal to be obtained. Saleeba et al. (1991), suggested the use of ^{35}S-labelled probes in the CCM reaction, which they claimed produced a cleaner more easily interpreted signal . This was attempted for the CF mutation and no real difference was seen . There was the advantage of safer handling during probe preparation but increased quantities of ^{35}S were required and this resulted in a much more expensive option.

Reports suggested that the CCM protocol should be able to detect the majority of mutations, but in this study, a variety of problems were encountered which rendered some mutations undetectable. Primarily , some of the exons in the *HGPRT* gene did not label well despite different labelling strategies employed. In particular exons 3 and 9 were problematic and this may have been due to the longer size of the amplification product. The PCR for exon 3 was not a robust PCR and the lower quantity of DNA obtained from the amplification of this exon coupled with the poor labelling, made CCM of this exon problematic. Often the uncleaved product could not

be seen or was so faint that it would have been doubtful if a cleavage product would have been detected.

The second major restriction was caused by non-specific cleavage events which occured in many of the *HGPRT* and *DMD* exons. This resulted in cleavage bands occuring across all lanes of a particular exon as is shown in figure 3.15 and often resulted in masking of the true cleavage product. This was particularly noticeable with cleavage of exon 7 of the *HGPRT* gene, where in most cleavage reactions a non-specific band was consistently seen below the non-cleaved probe band. If control lanes were included this band could be clearly identified and with some mutations such as HGPRT Marlow the cleavage product could still be seen. However with some exon 7 mutations such as HGPRT North Mymms, a cleavage product could not be seen. The most likely explanation could be that either the sequence of this exon is such that a secondary structure could be formed at the lower temperatures used for the modifications by hydroxylamine and osmium and that these modified bases are then later cleaved by piperidine. Therefore if these secondary structures are formed by hairpin loops it may be that the actual mutation is present within the loop and may miss modification. A second possibility is that the actual mutation is close to the falsely modified site and therefore after cleavage by piperidine the fragment is so small that it is run off the bottom of the gel. This phenomena was seen primarily with exon 7 but several other exons also showed non specific cleavage and this coupled with probe degradation during the protocol often made results difficult to interpret.

Other mutations such as HGPRT Mashad which involved an A to T substitution and hence a T.T mismatch could not be detected by CCM. T.T mismatches have been detected in other studies (Dianzani et al. 1991), and although rare T.G mismatches have been reported which are not reactive. Therefore the reason for non-detection is uncertain but the most likely explanation is that the heteroduplexes did not form correctly or that the quality of the test DNA was not particularly good. Therefore while CCM appeared a very efficient protocol it must be kept in mind that some mutations were not detected whether due to non-reactivity of the mismatch, non-specific cleavage or high background and that a proportion of mutations may be missed using this method of screening. Further the time consuming nature of the protocol coupled with the use of hazardous chemicals, again

limit the number of samples which could be handled at one time and the length of time required for exposure of the autorads was not practical for diagnostic use when large numbers of samples had to be analysed.

SSCP was not particularly effective in the detection of pathologies in the *HGPRT* gene. This was probably due to the large size of amplfication products containing the majority of the exons. The protocol did detect the mutations in the smaller amplification products, but it was not feasible at this point to design new primer sets for the entire gene which amplified small fragments, particularly when the HDHG protocol was working well with the existing primers. This method was therefore not fully evaluated at this point, but its potential role in the analysis of smaller amplification products and the ability of the protocol to analyse many samples at one time was noted.

4.1.3 Optimisation of DNA Sequencing Strategies.

Despite extensive optimisation, the manual sequencing of both the *HGPRT* and *DMD* exons was very problematic. A direct sequencing strategy had been established in the lab by Charles MgGone (personal communication), following the protocol suggested by Gyllensten and Erlich, (1988), but this strategy appeared to be unsuitable for the majority of exons of the *HGPRT* and *DMD* genes. The first step in the protocol involved the production of single-stranded DNA templates and despite alteration of the primer ratios, cycling parameters and templates used in the asymmetric PCR reaction, no products, single or double-stranded, could be obtained from a PCR reaction where one of the primers were limiting for the majority of exons. This was not due to the lack of quality or quantity of the double-stranded template as this allowed amplification when equal ratios of the primers were used.

Single-stranded DNA was obtained for exons 2, 4 and 6 and this probably reflects the robust nature of the PCR for each of these exons. The amplification of these exons very rarely failed and reasonable quantities of DNA were usually obtained. Further in the other mutation detection protocols these exons also produced good results. For exon 5 a smear was seen when the asymmetric PCR product was run on an agarose gel, but when this was sequenced no sequence was seen. The reason for the failure of the other exons to produce a product in this protocol may be primer or sequence

dependant, but also worthy of note is the fact that the exons which produced a product are generally the ones which are amplified in the smaller amplification products with the exception of exon 7, so both of these factors may be relevant. Further, use of sequencing primers internal to the amplification primers was essential to obtain sequence from the few exons possible.

Due to the failure of direct sequencing of asymmetric PCR products, other strategies were employed in an attempt to find the exact nature of the changes indicated by the other strategies. Primarily double-stranded PCR products were used as a template after chemical denaturation. This protocol had been successful for other groups sequencing double-stranded plasmids (F. Milan personal communication), but despite optimisation, no sequence could be obtained by sequencing of PCR products for any of the exons tested. This possibly reflected the quality or quantity of DNA obtained from the PCR product or the failure of the chemical denaturation. The probable explanation however is that that the structure of the PCR products, that of a linear double-stranded molecule, allowed renaturation of the denatured strands more quickly than a circular plasmid. This would explain the success of chemical denaturation when applied to a plasmid but not to a PCR product. A second approach was used in an attempt to obtain single-stranded product, involving the phosphorylation of one primer prior to PCR and subsequent digestion of that primer and amplified strand by a phosphatase to theoretically leave one single-stranded product. This was attempted and despite the presence of small quantities of poor sequence it was apparent that this strategy would not provide the sequence data required.

Even when sequence was obtained , it was generally of poor quality and often sequence of both strands could not be obtained. With some exons, in particular exon 4 sequencing was problematic due to the large stretches of homonucleotides in the gene. These long stretches of polyA caused the sequenase to stutter producing unreadable sequence thus rendering the elucidation of the insertion in exon 4 of the pedigree impossible. Clearly another strategy was needed for the sequencing of double-stranded PCR products thus circumventing the need to obtain single-stranded products and possible to obtain enhanced sequence from problematic exons.

Towards the end of this project access to an ABI automated sequencer became possible, and so it was decided to attempt to sequence some of the remaining DNA samples from the Lesch-Nyhan families using flourescent automated sequencing, coupled to a sequencing chemistry which negated the need for single-stranded templates. This technique, cycle sequencing, had been used for manual sequencing, by others using radioisotope and a protocol was available for coupling this with the use of fluorescently labelled dideoxy terminators so the sequence could be read by an automated sequencer. The protocol relies on repeated cycles of sequence elongation from primers, resulting in signal amplification which enhances the detection of the sequence ladder. An optimised cycle sequencing protocol was supplied by ABI which should allow the sequencing of the majority of sequences.

Different chemistries were available for automated sequencing, including the use of labelled primers or labelled terminators and in the use of Sequenase or Taq sequencing for analysis of single or double-stranded templates. Different chemistries have their benefits and drawbacks. Primarily the Taq terminator sequencing was chosen because a double-stranded PCR product could be easily used as a template without conversion to single-stranded DNA . This allowed the original PCR primers to be used as sequencing primers without the expense of designing new nested primers or biotinylated primers for single-strand manufacture on magnetic beads which is the alternative approach suggested. For a multiexonic gene the expense employed in having multiple sets of biotinylated primers manufactured, plus internal sequencing primers could be too high in a diagnostic envirament. By using the cycle sequencing dye-terminator approach, all four terminators are present in one tube and are labelled in different colours and there is no need for each of the four terminators to be added to separate reactions as is the case for dye-primers. Thirdly, difficulty has been reported in obtaining sufficiently high quantities of single-stranded DNA to obtain a high signal in the dye-primer approach, while with cycle sequencing and dye terminators, a smaller quantity of DNA is required due to the PCR amplification of the signal.

There are however drawbacks to the Taq terminator chemistry. Taq shows characteristic patterns of incorporation of dideoxynucleotides as it does not incorporate all ddNTP's with the same efficiency. This is

exaggerated by the presence of the dyes attatched to the ddNTP's as used in dye terminator reactions and the anomalous patterns are most pronounced in this chemistry. This is greatly dependent on the local base composition and sequencing artefacts can be created which are difficult to interpret. This study involved looking for the presence of heterozygotes, seen as double peaks. In this chemistry the peak heights are variable and equimolar concentrations of two bases at a heteozygous site do not always appear as peaks of equal hight. Background peaks which are more apparent in this chemistry also confuse the analysis , but artefacts can often be recognised by several sequencing reactions and by sequencing in both directions. There are some constant patterns which can be seen in chromatograms and when these patterns are recognised bases can be correctly assigned. These artefacts include C and T bases after G bases being much weaker signals and hence smaller peaks, and varying strengths of bases in strings of the same base. These and other patterns can be recognised and help to distinguish between true double peaks and artefacts. Other problems such as removal of unincorporated ddNTP's , cleaning up of the DNA templates and primers , gel artefacts such as unequal polymerisation , compressions and failure of sequencing due to bad DNA and primers still all occur.

The automated sequencing protocol was originally applied to the Lesch-Nyhan exon 2 which sequenced comparatively well using the previous strategies. This produced a good sequence and where DNA was available exon two from families with a mutation in that exon were analysed. The other exons were then sequenced used the same primer as was used for PCR with mixed results. Exon four could once again not be sequenced in either direction due to the strings of homopolymers after the primers, and exon five although producing a signal, still gave noisy sequence which was difficult to interpret. Exon three could be sequenced providing internal sequencing primers closer to the known mutation were designed. The protocol proved to be more effective than the manual sequencing, however the poor quality DNA and lack of DNA from several family members hampered a true analysis of the potential of this form of sequencing. Further, in cases where the mutation was known and the position and nature of the potential double peak was known, the presence or absence of the mutation could be seen. Where the mutation had not been previously identified it was problematic to identify a hetozygote from the background artefacts and no

new substitutions were identified in the *HGPRT* or *DMD* gene using this protocol. It was also easier to identify insertions or deletions in the heterozygotes as from the point of the base addition or subtraction the remaining bases were out of sequence and double peaks resulted along the length of the region.

This automated sequencing strategy was applied to the analysis of exons with band shifts in the *BRCA1* gene using the PCR primers as sequencing primers. Mixed results were obtained with some exons producing clean clear sequence and other exons proving difficult to sequence. However with increasing quantities of both template and primer and by designing a few internal primers most of these problems could be erradicated. Base insertion and deletions were again easier to identify but base substitutions were hard to notice against a background of unequal peak hights. The substitutions were easiest to detect when polymorphisms were present where the two species of homozygous sequence and the heterozygous sequence were sequenced and compared.

From our experience this automated sequencing was the best sequencing methodology available. Unfortunately by this stage there was no more of many of the Lesch-Nyah samples to confirm the heteroduplex detection, linkage analysis and CCM results. It was best applied to known mutations where the area of double peak could be expected therefore another mutation detection strategy to pinpoint the approximate location of the mutation once analysed by SSCP may need to be used.

4.2 Application of Screening Strategies to the Detection of Molecular Pathologies in Multi-exonic Genes.

After optimisation and evaluation of several mutation detection protocols, a strategy was needed for the detection of mutations in multiexonic genes. Many other groups have utilised a spectrum of mutation detection protocols and the most direct method appeared to be by analysis of the cDNA from these genes. This strategy had previously been applied to the analysis of the *HGPRT*, *DMD* and *BRCA1* genes and would decrease the workload of analysis of all exons individually as well as easily revealing where mutations had caused splicing aberrations due to the decreased size of the cDNA transcript. In this study however, this approach was not feasible

with the exception of one family, due the nature of the raw material available for analysis. The samples from the majority of the Lesch-Nyhan families available for the majority of the project, existed as old frozen blood, lymphoblastoid and fibroblast samples that had been temporarily thawed during transportation and at other stages. The material had therefore been damaged , and coupled with the fact that many of the lymphoblastoid and fibroblast cell lines tested were found to be contaminated by mycoplasma, (an *HGPRT* gene bearing organism), it was unlikely that RNA of sufficient quality could be harvested.

The majority of the boys with DMD/BMD were deceased and therefore the material available in stock, generally DNA, had to be utilised as little fresh samples could be obtained. Primer sets were also available for the amplification of the individual *DMD* exons so it was concluded that a strategy was needed for the analysis of multiple exons of the two X-linked genes.

The most readily availible source of material from the majority of the *BRCA1* families was again DNA and where RNA was available it was often unstable and cDNA's derived often did not sustain amplification. Further, primers had been designed and the PCR optimised for an individual exon to exon approach and different groups in the consortium manufacturd different primers so no one group had to manufacturedthe entire complement.

The X-linked nature of the first two diseases studied was both beneficial and detrimental. The X-linked location was beneficial as deleted portions of genes in males could be identified by PCR as there was no normal copy of the DNA from which amplification could occur and block detection as would occur with an autosomal gene. In the cases where no male DNA was available, maternal DNA had to be used. It is also beneficial because sequencing of male DNA produces sequence from only the affected allele and was not confused by the presence of the sequence from the normal allele . Heteroduplex analysis was however problematic as two alleles were required for heteroduplexes to be formed. This was easily rectified by the addition of control DNA to male DNA during PCR prior to heteroduplex detection, so that a potential heterozygous state could be established. A polymorphism in the control DNA was easily identified as all lanes would contain a doublet. In this instance, another control sample would be added to the test DNA.

The analysis of the mutation detection strategies revealed that in these experiments , heteroduplex detection using hydrolink gels was a more suitable strategy as a first line screening protocol in the *HGPRT* gene to determine which exon contained the mutation. This method allowed the detection of all tested mutations after modification, while SSCP analysis produced confusing results, did not detect all of the mutations and for the majority of mutations probably required digestion of the PCR products into smaller fragments for analysis or the manufacture of primers which amplified the exons in smaller segments. This further confused the results as well as increasing the cost and amount of work required. The second reason for the preference, was the relative safety and speed of the two protocols. Heteroduplex detection using hydrolink gels allows the loading of increased quantities of DNA into each well on the gel without loss of resolution so the bands can be easily detected by ethidium bromide staining. For SSCP analysis on acrylamide gel matrices the products were radioactively labelled for detection. Protocols have been suggested for non-isotopic methods of SSCP analysis but these proved to be technically difficult and unsatisfactory in our hands. Heteroduplex detection using hydrolink gels therefore proved to be more convenient rapid and safe due to the lack of radioactivity and hence the lack of need for time consuming autoradiography and radiation handling. Further, SSCP has been shown in several studies to be more efficient at detecting mutations in smaller fragments of DNA ideally of approximately 200-400bp in size. The majority of *HGPRT* exons were amplified in fragment sizes exceeding these parameters and heteroduplex detection on hydrolink gels has been shown to be able to detect mutations in fragments up to 1.3 kb in size which allows direct analysis of all exons analysed in this study without the need for restriction enzyme digestion. Due to the small number of exons in the gene and the comparitively low number of affected families the limitation of loading of only 10 samples per gel did not hamper this analysis, but it was apparent that with genes with a large number of exons and many samples to be analysed this could be a rate limiting step.

For detection of pathologies in cases where the mutation had not been identified, the DNA was extracted where required, though only a limited quantity of poor quality DNA was obtained from most samples. The strategy used for detection of mutations in the *HGPRT* gene, is outlined in figure 3.11. For the *HGPRT* gene, the individual exons with the exception of

exon 1 which could not be amplified, were amplified using PCR to identify the presence of large length mutations. Any bands of larger or smaller than expected size were then attempted to be sequenced to identify the exact nature of the insertion or deletion. Lack of amplification of an exon indicated the deletion of a whole exon or part of an exon of the gene which are reasonably common in Lesch-Nyhan syndrome.

The majority of mutations in Lesch-Nyhan syndrome result from a heterogeneous collection of point and small length mutations, (Davidson et al., 1988,1989, 1991; Gibbs et al., 1989; Gibbs et al., 1990; Marcus et al., 1992; Yamada et al., 1992; Steingrimsdottir et al., 1992; Sege-Peterson et al., 1992; Tarle et al., 1992, Fujimori et al., 1991). Over 100 mutations have been described which cause Lesch Nyhan syndrome and *HGPRT* associated gout. A notable feature of the list is the great variety of mutations that can cause Lesch-Nyhan syndrome with mutations being present along the entire length of the gene. Many of these mutations have been documented in the computer databases. 61 such mutations were documented in 'The Mendelian Inheritance in Man' and these appear to be representative of the types of pathologies found in this gene for *HGPRT* deficiency diseases. Of these mutations described 65.5% of the mutations were single base substitutions which lead to single amino acid substitutions or the introduction of premature stop codons which results in the production of a truncated protein. Approximately 8% of the mutations were the result of base insertions into the *HGPRT* gene and 23% were due to deletions of variable sizes including some of 1bp leading to frameshifts and premature stop codons and some of whole exons or indeed the entire *HGPRT* gene. The remainder of the mutations were complex gene rearangements involving insertions, and deletions which altered the entire structure of the coding sequence and hence the protein. Many of the pathologies resulted in abberant splicing of the *HGPRT* gene and while many of these were discovered as a result of examination of the cDNA from patients, these changes should also be apparent if the intronic sequences adja-cent to the exons are examined in cases where no RNA is available.

Due to the wide range of mutation types found in this gene and the presence of pathologies reported along the length of the gene and in the intronic sequences, a strategy was required to screen for large and small DNA changes. After PCR analysis had excluded large aberrations, the

exons were screened for the presence of small changes. This was done primarily by heteroduplex detection on hydrolink gels, with each sample being analysed several times by using at least two different PCR reactions with at least two different control samples used. The samples were run at different voltages on different lengths of gel with the guidelines learned from the optimisation of the protocol using known mutations in the different exons used . However, despite the apparent success of heteroduplex detection on hydrolink gels in this study, extensive optimisation was required for each mutation and due to the lack of information from other groups as to the percentage of mutations detected, it has to be presumed that some changes may have been missed by this protocol due to failure of the PCR to amplify both alleles or due to the failure of the gel matrix to separate the different duplexes. For this gene therefore all the exons from the 13 families were also examined for base changes by CCM analysis using both hydroxylamine and osmium tetroxide to modify all potential bases before cleavage with piperidine. Once more information is available as to the effectiveness of the hydrolink protocol only the exons deemed suspicious by this protocol would be screened by CCM analysis.

Due to the nature of the CCM protocol, a certain degree of probe degradation occurs and this often potentially obscured the identification of cleavage products. While CCM is a potentially ideal method for the theoretical detection of 100% of mutations there are many technical difficulties as was described, which can cause difficulties in the interpretation of results. Also due to the complex nature of this time consuming, hazardous, multi-stage protocol, human and technical errors involving such factors as fluctuations in temperatures of water baths or the preferential formation of homoduplexes, or even deficient labelling of probes, failure of the protocol cannot be excluded and some mutations may be missed. Once again, the time consuming nature of the protocol and the limitation in the number of samples which can be handled at one time, limits the value of this protocol for large genes, where many samples need to be analysed.

Using the strategy outlined for detection of mutations in the *HGPRT* gene, four mutations were definitively found in the families studied. Three of these could be sequenced the first of which being a G-A substitution in exon 2 in the index case from pedigree Gla 7292. This mutation resulted in an amino acid substitution of lysine for arginine at amino acid 45 and as the

mutation occurred in the last coding bases of exon 2 , may interfere with the splicing of the gene. No RNA was available to confirm this finding. This type of substitution mutation is the most common found in the *HGPRT* gene and this mutation is fairly representative of the type of mutations most commonly found. The second mutation , a T deletion in exon 6 of the index case from the pedigree Gla 7291 results in a frameshift mutation leading to the production of a truncated protein. Deletions of one base to entire portions of the gene are common in Lesch-Nyhan syndrome and again this type of mutation is fairly representative of the type of mutations which would be expected to be found. The third mutation discovered however, was more unique, a 60 bp insertion in the cDNA of the index case from the pedigree10427. The DNA from this index case could not be sequenced as it was difficult to obtain an amplification product from this region of the DNA. After sequencing the cDNA, the sequence was compared to a sequence database and an Alu sequence had been inserted into the cDNA between exons 6 and 7. There have been no previous reports of the insertion of only an Alu sequence into the *HGPRT* gene, but there has been one report of the duplication of a region of the gene which arose through unequal homologous recombination betwee two Alu sequences and involves a much larger duplication of some 1.8kb (Marcus et al.,1993). The origin of the Alu sequence and the exact point of theinsertion could not be further characterised as the DNA could not be sequenced. The fourth mutation, a large insertion in exon 4 of the index case from the pedigree 7293, could not be sequenced but the presence of the insert could be clearly identified in family members. DNA was not available from other family members from two of the pedigrees but if these are provided in the future the mutations can be clearly identified by the methods described.

Over 70% of the pathologies in the DMD gene were large deletions of one or more of the exons of the gene. The index cases with DMD in this study had previously been screened for deletions and the remaining index cases where no deletions were detected were screened for the presence of smaller changes such as small insertions, deletions and substitutions. The majority of the small changes previously reported in the gene caused the production of truncated proteins and the ideal scenario would have been to carry out protein truncation analysis on cDNA from the index cases. However no RNA was available for any of the index cases and as many had died, the only material available for analysis was DNA. Due to the

increased number of exons in the *DMD* gene, it would have been too expensive and time consuming to analyse all the exons where PCR primers were available (21 in total) from every individual with both hydrolink and CCM strategies. Therefore only the exons which yielded suspicious results after heteroduplex detection were analysed using CCM.

The ideal protocol would result in the sequencing of suspicious exons to identify the exact nature of the mutations. This stage of the strategy was the weakest link in the chain as the manual sequencing strategies were problematic for both genes as was described. At this stage access was not availible to an automated sequencer. Small amounts of poor sequence was obtained and even after extensive optimisation it was very difficult to obtain adequate sequence information for many of the potential mutations. Added to this is the potential hazard of introduction of base changes by the PCR protocol itself. Consequently although potential mutations were present in many of the families these could not be conclusively identified at this stage. Many of the DNA samples were finished or degraded by the advent of the automated sequencing strategy and in future mutations found in other genes would be sequenced in this manner.

Primer sets were available for 21 exons of the DMD gene and in some of the individuals, 9 of these exons had previously been analysed by SSCP analysis by Kataki, (1992) and no mutations had been found. Due to the fact that SSCP was found to detect only a percentage of known mutations analysed in this study and that many false positives were reported, these exons from these individuals as well as all the exons from individuals where no analysis had been carried out were screened using HDHG and CCM analysis in an attempt to find small mutations or polymorphisms which may have been present.

Primer sequences for the 21 exons were available (Chamberlain et al., 1988, Beggs et al., 1990, Abbs et al., 1990), and the conditions for the amplification of individual exons using primer sets a-i had been optimised by Kataki, (1992). The PCR reaction for the amplification of the remaining exons individually was easily optimised in this study and the PCR reactions for the majority of the *DMD* exons were very robust and few spurious bands were seen. The primers for amplification of exons a-i were however quite old and during the course of the study amplification failure occured but could

usually be re-established either by increasing the quantity of the primer used in each reaction or by cleaning of the primer by precipitation.

HDHG was used as the initial screening strategy and as for the *HGPRT* gene, control DNA was added to the PCR reaction to create a potential heterozygote state. All the amplification fragments were of a small to medium size and the hydrolink gels run were 20cm in length. The HDHG protocol was straightforward and the robust nature of the PCR reaction provided a good quantity of clean DNA for heteroduplex analysis. However, in several exons in particular exons 43 and 50 the results were unclear as when the PCR product was run on a hydrolink gel a second band was consistently present above the first in all lanes which was not seen on agarose gels.

The exons which showed a positive or suspicious HDHG result were then analysed by CCM analysis using both hydroxylamine and osmium tetroxide . This appeared less problematic than for the *HGPRT* gene as the robust PCR allowed good labelling of the probes and less background on the autorads. There were however still some non-specific cleavage products for some exons which hindered the analysis of the results. Lack of DNA or primer sets hindered the analysis of the exons from some index cases. However the CCM analysis confirmed the HDHG results.

As with the *HGPRT* gene, the direct sequencing of the PCR products from the asymmetric PCR proved to be problematic and single-stranded products could not be obtained for many of the exons despite applying all the optimisations previously described. Sequence could not therefore be obtained by this methodology to confirm several of the changes found. The changes found in this gene could have been polymorphisms and as these have been reported within the *DMD* gene it could not be presumed that any changes found were the molecular pathologies responsible for the disease. Internal primers were not available for any of the exons due primarily to the lack of genomic sequence information available at the time of analysis and to the quantity of exons being screened. Double-stranded sequencing was also applied to several exons but once again no sequence was obtained. Sequence was obtained for two of the particularly suspicious exons which showed both positive HDHG and CCM results , namely exon a from pedigree 6397 and exon b from pedigree 5117, but many stops and

compressions were present and this may have led to any mutations which were present being masked. The results of the screening for pathologies in the *DMD* gene were very disappointing and the possibility remains that the large size of the gene is not suitible for analysis of the DNA exon by exon. Other strategies which look at larger portions of the gene at one time such as protein truncation studies, seem to have been succesfull and are probably a better way to look for mutations in this gene.

After the discovery of the *BRCA1* gene by Miki et al., (1994), Many mutations were reported in the *BRCA1* gene in families with early onset breast/ovarian cancer. (Castillla et al., 1994; Friedman et al., 1994; Simard et al., 1994; Shattuck-Eidens et al., 1995). These mutations are reported along the length of the gene with the only clustering of the mutations appearing to be in the RING finger motif, which while only encoding for 2.3 % of the protein, contains 11% of the mutations so far described. Shattuck-Eidens et al., ((1994) published a collaborative study detailing the mutations found in the *BRCA1* gene by groups throught the world and from a study of these mutations coupled with other mutations subsequently described, it appears that the majority of *BRCA1* pathologies (79% in the Shattuck-Eidens study) are small frameshift or nonsense mutations resulting in truncated or unstable proteins. The possibily must however be considered, that these mutations may be the easiest to detect using the methods so far chosen to look for mutations and due to the autosomal nature of the gene , deletions of entire exons of the gene may be missed when DNA is analysed as the normal copy of the gene from the other chromosome can be amplified and mask the deletion. Several of the mutations described have been found in unrelated familes representing possible common mutations which can be screened for as a first step in any screening strategy. 38% of the original mutations found are recurrent and these mutations account for approximately 63% of the total mutations detected in the Shattuck-Eidens(Shattuck-Eidens et al., 1995) study and indeed a large proportion of the pathologoes more recently reported are due to one of these mutations.

Due to the large size of the *BRCA1* gene and the fact the the gene was amplified in over 39 segments, coupled to number of samples for analysis(60 family member samples, 100 sporadic tumours and 50 ovarian tumours) it was not feasible to analyse this gene using HDHG analysis. The primers for this gene were designed to produce products of less than 300bp,

so it was decided that SSCP analysis coupled with an automated sequencing strategy would be employed. Further, this was the method employed by the majority of groups looking for *BRCA1* mutations. The exons were amplified and the gels run under two conditions, at room temperature and at 4^{0}C, in order to obtain maximum efficiency of detection. Each gel could run with 48 samples and any shifts seen were sequenced. Many band shifts were observed and all were scanned for the presence of heteroduplex bands in the sequence chromatograms, keeping in mind the limitations previously discussed. The blood samples were analysed, where any changes would probably be in a heterozygous state, while in the tumour samples loss of heterozygosity would result in the loss of the normal allele. Many fragments exhibiting a band shift showed no obvious changes and it is uncertain if this is due to the high false positive rate of SSCP analysis or to the failure of the sequencing strategy to show these mutations. Substitutions were harder to identify than insertions or deletions and in cases where known mutations were being sought, it was easier to detect the changes when the location was known. However, this strategy seemed good for this gene and four mutations were discovered in seven families and three polymorphisms were identified. Not all areas of the gene could be analysed at this point, so the strategy aimed the detection to regions of the gene previously shown to contain mutations. This included exon 11 which was the first region to be analysed and exons 2, and 20 and intron 5, which were shown to contain common mutations. Two of the common mutations the C insertion in exon 20 and the AG deletion in exon 2 each resulting in the production of a truncated protein, were each found in one of our families and a third mutation, an AA deletion in exon 20 , which had been described by Friedman et al (1994) in one family, was found in four Scottish families possibly indicating a common Scottish mutation. This mutation resulted in a truncated protein and if it appears to be relatively common in the Scottish population , antibodies may be able to be made against the protein sequence after the frameshift and this mutation may be able to be found on the bases of antibody recognition. The fourth mutation was an A-G substitution in exon 2 resulting in an Ile to Val amino acid substitution. While these amino acids are very similar, the position of the substitution, at the second base of the RING finger motif may reflect its pathological importance. This mutation highlights one of the problems associated with mutation detection and hence carrier detection in this gene. The function or structure of the protein is not yet fully understood and it is very difficult to determine

the importance of many of the mutations found. Many single base substitutions could be polymorphisms that track with the disease through a family and hence base substitutions found need to be looked for in the population. This mutation in our family was not present in any of the other BRCA1 families or in any control DNA analysed but untill the function and structure of the BRCA1 protein is further characterised no further information can be gathered about this mutation and many others reported by other groups. Richard and Beckmann(1995) reported a silent third base mutation in the calpain gene(*CANP3*), a gene associated with chromosome 15-linked limb girdle muscular dystrophy. This apparent neutral polymorphism led to the production of an abnormally sized transcription product. They demonstrated that this mutation was actually a gain of donor splice site mutation. Computer programmes failed to predict the creation of this splice sight which could only be demonstrated by the analysis of illegitimate transcripts from the gene. Other such mutations have been reported and these pathologies highlight the potential for any base change found in affected families to be significant and that these changes may be further characterised by analysis of the gene trascripts. In the remainder of families a portion of the gene was still to be analysed, but it is uncertain whether many of these families are linked to the *BRCA 1* gene, as clinics sent the DNA purely on the basis of a positive family history which could be due to the clustering of sporadic cases or to another familial gene. Further, linkage analysis was not possible in the majority of these families as DNA from key family members was not available. Even in families where linkage has been positively attributed, it is impossible to rule out the possiblility that females within the families may have sporadic breast /ovarian cancer and that the index cases examined for mutations in these families may be such cases and hence no mutations will be found.

For the majority of the tumour DNA samples where no *BRCA1* mutation has yet been identified, the mutation may be present in regulatory regions of the gene not yet analysed or these mutations may be present in other regulatory genes or other genes in the region not yet identified, which may be downstream in the *BRCA1* regulatory pathway. The methodology used to detect the mutations may be missing a proportion of the changes and other strategies such as the protein truncation test (PTT) which was used by Hogervorst et al ., (1995) succesfully to find several novel *BRCA1* mutations may need to be employed in conjunction with other technologies.

As the majority of breast cancers are thought to be sporadic it would be important to ascertain if *BRCA1* has a role in the initiation or progression of sporadic cancers. Several other cancer predisposing genes , such as the *Rb* gene and the adenopolyposis coli gene, have been shown to be initiators of the tumourigenic process and in inherited disease the affected individual inherits one mutated copy of the gene through the germline and acquired the second mutation to the other copy. The observation that many sporadic breast and ovarian cancers also show loss of an allele in the BRCA1 region, support the potential role for the *BRCA1* gene in these cancers (Futreal et al., 1992, Saito et al., 1993,). Although Futreal et al., (1994), examined sporadic breast/ovarian tumours with LOH , only a small percentage contained mutations in the *BRCA1* gene and these mutations appeared to arise in the germline. Recently, mutations have been reported in sporadic ovarian cancers (Merajver et al. 1995, Hosking et al. (1995). No mutations have as yet been identified in sporadic breast cancers indicating that *BRCA1* mutations may have no role in the development of these cancers. In this study we were unable to look for mutations in the majority of the sporadic breast and ovarian cancer DNA's. Most of our material was provided as blood samples from genetic clinics, and in sporadic tumours the mutation would not be present in the blood ,only in the tumour. However some of the cases detailed as sporadic could have been due to familial cancer cases and hence to mutations in the *BRCA1* gene. These presumed sporadic breast and ovarian cancer blood samples were analysed by ASO hybridisation for the common mutations, but none were found in any of the samples.

4.3. The Application of Mutation Detection Strategies to the Detection of the Mutant Gene in Lesch-Nyhan Familes.

DNA was available from several female family members of index cases with Lesch-Nyhan syndrome, but for females, clinical evaluation is not a reliable form of carrier determination and the biochemical analysis of female carriers is problematic. Carrier detection is most reliable where DNA analysis is diagnostic, and this can be done using linkage analysis or by direct detection of the mutation.

Linkage analysis has been used for many years to track the path of a disease carrying chromosome or gene within a pedigree. This can be done

using conventional RFLP analysis or if a tandemly reiterated sequence which is highly polymorphic is available at a close proximity to the gene being traced, this can be a valuable and stable marker. One such hypervariable short tandem repeat (STR) reported within the *HGPRT* gene (Edwards et al. 1991), contains a tandem AGAT tetranucleotide repeat motif present in 9-16 copies. It is stably inherited and can be easily amplified by PCR using flanking primer sequences (Edwards et al. 1991).

This STR was used in this study to attempt to crudely determine the carrier status of female relatives of boys with Lesch-Nyhan syndrome. The PCR-STR approach was used in nine families where DNA was available from a sufficient number of key family members. In six of these families the alleles were informative and carrier status could be assigned as is shown in figure 3.23. In three of the families however the STR was uninformative due to the maternal alleles being non-informative or to the lack of DNA from key family relatives.

This PCR based strategy was very robust and has several advantages over conventional RFLP, Southern blotting, in the analysis of this disease. Often very small quantities of partially degraded DNA was available and amplification of the DNA by PCR negated the need for larger quantities of DNA that would be required for Southern Blotting after restriction enzyme digestion. The PCR-STR approach also provided results more quickly than Southern blotting and the position of the STR within the *HGPRT* coding sequence also reduced the likelihood of false results due to recombination between the gene and the marker.

There are several limitations however to this form of indirect carrier detection. As with conventional RFLP linkage analysis, DNA has to be available from key family members and this DNA is not always readily available. In many cases the alleles from the missing people can be determined by looking at the alleles present in the other family members, particularly the children as was the case with family 10185 (figure 3.23.e). In this pedigree the grandmaternal and grandpaternal alleles needed to be determined to see whether the aunt of the index case had inherited the chromosome which tracked with the affected allele. However this is not always possible and the absence of DNA from key family members hindered the application of this methodology in many of the families . Non-paternity is

also a problem in this and conventional linkage analysis. In one pedigree 10195 , where the maternal allele is not informative, the allele sizes clearly show that one daughter has not inherited one of her alleles from either parent . This suggests non-paternity and puts a question mark on the paternity of the other family members. In this case the allele which was not inherited from the father was clearly visible but in other cases where paternal DNA is not available or one of the paternal alleles cosegregates with the maternal allele, the entire diagnosis could be jeopardised. Varying figures have been quoted for rates of non-paternity , with levels quoted as high as 10%. This is therefore a potential pitfall of this type of carrier detection .

Following the segregation of alleles in a family can determine clearly which female family members are not carriers, barring the possibilities of recombination and non-paternity already discussed. However if a female is shown to have inherited the same allele and hence the same chromosome as an affected index case, it cannot be definitely concluded that she is a carrier. This is due to the 1/3 rate of new mutations in affected index cases . Only 2/3 of mothers of affected boys will theoretically carry the mutation which may be a new mutation in her germline cells or in her parents. The exact origin of a mutation in a pedigree can never be determined by linkage analysis so a percentage of female relatives will be falsely assigned as carriers. Some of the aunts of affected boys in this study could have their carrier status clarified by looking at the grandparental alleles to identify whether the mother has inherited the grandpaternal or grandmaternal allele. If she has inherited the grandpaternal allele, then as he only has one X chromosome and is not affected by the disease, it suggests that the new mutation is in the mothers germ line and could not be present in any of her sisters barring the possibility of gonadal mosaicism.

Even after all of the disadvantages of this system have been considered, it it clear that in this gene, linkage analysis using this marker has been very successful as a first step in the determination of carriers. This technology could therfore be applied to genetic counselling in families which were informative, but a more satisfactory result could be given when this was coupled to direct mutation detection and the actual pathology shown to be present or absent.

Consequently, direct mutation detection strategies were employed in families where DNA was available and HDHG , CCM and sequencing analysis were applied to each family. Ideally both protocols should have provided information in each family and a definite carrier assignment made.

However, due to some of the deficiencies outlined previously, this was not the case and in many families definitive results could not be obtained for some of the protocols . In only four of the families were both strategies informative and both CCM and HDHG results indicated carrier status in all female relatives available. In a further family CCM and HDHG were not needed as a large insertion in exon 4 of this family allowed carriers to be directly assigned by running the PCR product on an agarose gel. In the pedigree 10427 the presence of a 60bp insertion in the cDNA could also be indicative of carrier status in female family members, which were not provided to us. In the two families analysed where the molecular pathology was in exon 7, one mutation was clearly identified by CCM and the other was not. Unfortunately the mutation was identified in two brothers with gout who had the mutation HGPRT Marlow and as they had the disease, it was already clear that any daughters would be carriers, therefore negating the need for carrier detection. In the other family with the mutation HGPRT North Mymms, the mutation could not be detected by CCM, probably due to the presence of the non-specific cleavage product which was masking the true cleavage product. This mutation could however, clearly be identified in the index case by HDHG analysis, and appeared to be absent in the DNA from female family members indicating that it was a new mutation in the index case. The sequencing results from the automated sequencer support the HDHG results, although no more DNA was available from the index case. So despite one sister(3, figure 3.26) appearing to have the same maternal chromosome as the index case in the linkage analysis, it appears that neither she nor the mother is a carrier

CCM also failed to detect the mutation in exon 2 from the pedigree 10197 (HGPRT Mashad), while HDHG and sequencing results, indicated that all the sisters were not carriers. HDHG also clearly allowed carrier assignment to the mother and aunt of the index case from the pedigree 10199(HGPRT Runcorn) though a limited amount of DNA was available for this family and neither linkage analysis or CCM could be carried out to confirm this data.

For the pedigree 10198 (HGPRT Codicote), linkage analysis was uninformative, and insufficient maternal DNA was available for direct mutation detection protocols. The mother was however an obligate carrier, due to the detection of HGPRT deficiency in a second male fetus which had been terminated. The DNA from the sister and aunt showed no heteroduplex band or cleavage product and although no DNA was available for sequencing of the index case , no T insertion appeared to be present in these two individuals. These results taken together indicate that these females are not cariers and other female family members could easily be screened when DNA samples become available.

In pedigree 10186, the mutation,a G to C substitution in exon 3 , caused an alteration in a restriction enzyme sight and no mutation detection strategies were required as the carriers could be assigned by presence or absence of the cut at the restriction enzyme site (S. Laughlan, personal communication). This was the ideal situation as mutations which alter restriction sights can be easily identified without the need to undertake other time consuming protocols.

The results of the direct mutation analysis on these families was disappointing . In some of the families no further information could be given beyond linkage analysis as the mutations were previously unknown and could not be fully characterised due to lack of sequence information. In other families CCM proved problematic due to insufficient probe labelling or high background signal due to probe degradation. While HDHG proved very reliable when definite mutations were being sought in index cases, the interpretation became more complex when the presence of the mutation was uncertain . This would be satisfactory if CCM and sequence information confirmed the data, but carrier assignment would not be given on the strength of this alone. If the direct sequencing protocol could have been optimised this would have provided the definitive carrier designation and the way forward for more comprehensive carrier designation would be to find a suitable strategy for obtaining consistant sequence for all exons. The automated sequencing protocol did however provide further information in five families. This coupled to the fact that the sequence information agreed with the HDHG and CCM data, made the data reliable and carrier status could be further confirmed.

As no mutations were identified in the *DMD* gene no carrier detection could be carried out on female family members.The large size of this gene may indicate that the strategies examined here are not suitable for such a large gene and that possibly examination of the cDNA to reveal abnormal transcripts or the use of strategies to analyse the protein such as protein truncation studies may be more suitable. At this stage it was not feasable to do carrier detection for mutations found in the *BRCA1* gene. Most detection of the pathologies in Scotland at this stage were carried out in a research environment and no definite plan for screening family members has yet been implemented until many of the problems associated with screening in this disease have been dispelled.

CHAPTER 5

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APPENDIX 1

SOLUTIONS

All solutions were prepared with distilled water and are listed alphabetically.

2X annealing buffer(CCM) : 12mM Tris-HCl pH 7.7, 1.2M NaCl, 14mM $MgCl_2$. The solution was autoclaved and stored at room temperature.

5X annelaing buffer(sequenase 2. kit USB): 200mM Tris-HCl pH 7.5, 100mM $MgCl_2$, 250mM NaCl. Stored at -20^{0}C.

Chorion buffer : 50mM Tris pH7.5, 100mM NaCl, 1mM EDTA,.

Denhardt's solution : 2%(w/v) BSA, 2%(w/v) Ficoll, 2%(w/v) polyvinylpyrolidone

dNTP mix(Boeringer): A stock solution of dNTP's was made up by mixing 5ml of each dNTP (dATP, dCTP, dGTP and dTTP), with 380ml of double distilled water. Stored at -20^{0}C.

'HOT' stop solution : 0.3M sodium acetate pH 5.2, 0.1mM EDTA, 25mg tRNA/ml. Solution autoclaved before addition of tRNA and stored at -20^{0}C.

Hydroxylamine solution : 1.39g hydroxylamine in 1.6ml ddH2O, adjusted to pH 6.0 by addition of diethylamine. Solution made up on day of use.

Kinase buffer : 0.5M Tris-Cl pH 7.6, 0.1M $MgCl_2$, 50mM DTT, 1mM spermidine, 1mM EDTA.

Loading mix1, for acrylamide gels and SSCP gels : 95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol.

Loading mix 2, for agarose gels. 0.25% bromophenol blue, 0.25% xylene cyanol FF, 40%(W/V) sucrose in water.

Loading mix 3, for hydrolink gels: 5% sucrose, 0.06% orange G, in water.

Loading mix for automated sequencer gels : 1part (20mM EDTA, 30mg blue dextran): 5 parts diionised formamide.

Lysis buffer : 0.32M sucrose, 10mM Tris-Cl pH 7.4, 5mM $MgCl_2$, 1% Triton X-100. The solution was autoclaved and stored at 4^{0}C.

NEW solution(Geneclean Kit) : 0.5ml concentrated NEW solution (supplied with kit), 10ml ddH2O, 11.1 ml 100% ethanol(filtered). Stored at -20^{0}C.

Nuclei lysis buffer : 10mm Tris-Cl, 0.44 M NaCl, 2mM EDTA pH 8.2. The solution was autoclaved and stored at room temperature.

OsO_4 buffer : 10mM Tris-HCl pH 7.7, 1mM EDTA, 1.5% pyridine. Solution stored in safety cabinet in a darkened container.

OsO_4 solution : 0.5g/12.5ml ddH_2O left to dissolve for 48hours before use. Solution is stored in a safety cabinet in an airtight darkened container and is made up into a 1/5 dilution on day of use.

10X PCR reaction buffer(Boeringer).

Phenol/chloroform : 50% phenol, 50% chloroform, 0.1% 8-hydroxyquinoline equilibriated to pH 7.5 with T.E. buffer. Topped with buffer and stored in a dark bottle at 4^0C.

10% piperidine : 100ml piperidine in 900 ml ddH_2O. Solution stored as 100% stock solution in a darkened bottle in a safety cabinet and is diluted on day of use.

Prehybridisation buffer for ASO dot blotts : 3.0M TMAC, 0.1M $NaPO_4$ pH 6.8, 1mM EDTA, 5X Denhardt's solution, 0.6% SDS, 100µg/ml yeast tRNA.

20X SSC : 3M NaCl, 0.3M sodium citrate.

Stop solution (Sequenase 2 kit, USB) : 95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF. Stored at -20^0C.

10X TBE buffer (5 litres) : 540g Tris base, 275g boric acid, 200ml 0.5M EDTA pH 8.

T.E. buffer : 10mM Tris-Cl pH 7.4, 1mM EDTA. Solution was autoclaved and stored at room temperature.